MULTI-LEVEL REGULATION OF PHENYLALANINE HYDROXYLASE IN *Pseudomonas aeruginosa*

By

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Dedicated to my father and my mother,

whose love, care, and encouragement make it possible

for me to complete this dissertation

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Abstract of Dissertation Presented to the Graduate School of the University of Florida in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

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Pseudomonas aeruginosa was recently found to possess a cluster of genes (*phh* operon) encoding phenylalanine hydroxylase (PhhA), 4**a**-carbinolamine dehydratase (PhhB), and aromatic aminotransferase (PhhC). In the flanking upstream region of the *phh* operon, a divergently transcribed gene (*phhR*) that encodes an activator protein was identified. Inactivation of *phhR* markedly reduced expression of the three structural genes. PhhR belongs to the large prokaryote family of σ^{54} enhancer-binding proteins, and activation of the *phh* operon by PhhR in *P. aeruginosa* required *rpoN*. *P. aeruginosa* PhhR was able to replace *E. coli* TyrR as a repressor of the *aroF-tyrA* operon (but not as an activator of *mtr*) in the heterologous *E. coli* system. The *phh* operon was strongly induced in fructose- or glucose-based minimal medium by *L*-phenylalanine and *L*-tyrosine, and less by *L*-tryptophan.

Inactivation of *phhR* in *P. aeruginosa* abolished ability to utilize either *L*-phenylalanine and *L*-tyrosine as a sole source of carbon for growth.

PhhB is a bifunctional protein. It was shown to have 4**a**-carbinolamine dehydratase activity as well as regulatory activity. The expression of *phhA* was activated by the presence of *phhB* in both *E. coli* and *P. aeruginosa*. Transcriptional and translational fusion analysis showed that the regulatory effect of PhhB on the expression of *phhA* is at the post-transcriptional level.

An insertionally inactivated *phhB* mutant failed to grow on *L*-phenylalanine or *L*-tyrosine as a sole carbon source. Expression of PhhA in the absence of PhhB causes strong growth inhibition in *E. coli*. The inhibitory effect is probably caused by 7-tetrahydrobiopterin, which is known to be formed in the absence of PhhB. Since 7-tetrahydrobiopterin is a potent inhibitor of phenylalanine hydroxylase, this could account for the inability of *phh*A in the absence of *phh*B to complement *E. coli* tyrosine auxotrophy. The general inhibition of growth may be due to inhibition of some unidentified essential pterin-dependent enzymes.

CHAPTER 1
LITERATURE REVIEW

Phenylalanine Hydroxylase in Nature

Phenylalanine hydroxylase (phenylalanine hydroxylase 4-monooxygenase; EC 1.14.16.1) catalyzes the irreversible conversion of *L*-phenylalanine to *L*-tyrosine (Kaufman, 1987). In mammals this enzyme catalyzes the initial, obligatory, and rate-limiting step in the complete catabolism of serum phenylalanine to CO_2 and H_2O (Kaufman, 1986). A deficiency of this enzyme causes accumulation of serum phenylalanine, leading to hyperphenylalanemia. Because metabolism of phenylalanine is restricted to alterations in the alanyl side chain of phenylalanine, in the absence of phenylalanine hydroxylase, the formation and excretion in the urine of compounds such as phenylpyruvate and phenyllactate occurs. This condition is called phenylketonuria, a genetic disorder associated with severe mental retardation in untreated children (Dilella *et al.*, 1986). Many mutations at the phenylalanine hydroxylase locus have been identified (Guldberg *et al.*, 1996).

Phenylalanine hydroxylase has been intensively studied in mammals for many years. It is a member of a family of enzymes that also includes tryptophan hydroxylases (EC 1.14.16.4) and

tyrosine hydroxylases (EC 1.14.16.2). All three enzymes utilize a tetrahydrobiopterin cofactor and molecular oxygen to hydroxylate their respective aromatic amino acid substrates (Kaufman and Fisher, 1974). Phenylalanine hydroxylase has been purified from rat liver where it is an oligomeric protein (predominantly homotetramers) composed of 52-kDa subunits (Davis *et al.*, 1996). It has non-heme iron as the active-site metal. The rat liver hydroxylase was also expressed in *E. coli* and purified to homogeneity (Kappock *et al.*, 1995). The homotetrameric recombinant rat hepatic phenylalanine hydroxylase is highly active and is identical to the native enzyme in many properties.

Although mammalian phenylalanine hydroxylase has been intensively studied, few studies on bacterial phenylalanine hydroxylase have been done. Phenylalanine hydroxylase has generally been considered to be of rare occurrence in prokaryotes, where scattered reports of its existence have been limited to one phylogenetic division of gram-negative bacteria. They include *Pseudomonas acidovorans* (previously known as *Pseudomonas* sp. ATCC 11299a) (Guroff & Ito, 1963). *P. facilis* (Decicco & Umbreit, 1964), *Alcaligenes eutrophus* (Friedrich & Schlegel, 1972), and *Chromobacterium violaceum* (Letendre *et al.*, 1974). Of the three pterin-dependent and metal-containing hydroxylases, only phenylalanine hydroxylase from *Pseudomonas acidovorans* (Letendre *et al*, 1975) and *C. violaceum* (Nakata *et al.*, 1979; Pember *et al.*, 1986) has been

purified and characterized. The *C. violaceum* phenylalanine hydroxylase gene was the first one to be cloned and sequenced from a bacterium (Onishi *et al.*, 1991). High identity of the deduced amino acid sequence with those deduced for the mammalian hydroxylase gene family was found and showed that the microbial hydroxylase and the mammalian hydroxylases are homologous. Although *C. violaceum* phenylalanine hydroxylase is a pterin-dependent enzyme, it differs from the mammalian enzymes in its smaller subunit size (lacking the N-terminal domain responsible for the complex regulation in the mammalian enzymes), its existence as a monomer (rather than a homotetramer), and binding of copper (instead of iron) at its active site. However, the surprising claim has been advanced that *C. violaceum* phenylalanine hydroxylase does not require any redox active metal for its activity (Carr & Benkovic, 1993; Carr *et al.*, 1995).

P. aeruginosa belongs to a different superfamily of gram-negative prokaryotes than do the aforementioned organisms. It was found to possess homologues of mammalian phenylalanine hydroxylase, 4**a**-carbinolamine dehydratase/DCoH, and aromatic aminotransferase as part of a three-component gene cluster (Zhao *et al.*, 1994). These three genes are *phhA, phhB*, and *phhC*, respectively. The *P. aeruginosa* phenylalanine hydroxylase contains iron and is pterin-dependent. Unlike the multimeric mammalian hydroxylase, the native *P. aeruginosa* hydroxylase is a monomer.

The Pterin-Recycling Enzymes

Phenylalanine hydroxylase catalyzes the conversion of *L*-phenylalanine to *L*-tyrosine, using tetrahydrobiopterin as a reducing agent and relying upon molecular oxygen as an oxidizing agent (Kaufman, 1987). During this hydroxylation reaction, the tetrahydrobiopterin cofactor is stoichiometrically oxidized to a carbinolamine, 4**a**-hydroxytetrahydrobiopterin. Two essential enzymes, 4**a**-carbinolamine dehydratase and dihydropteridine reductase, are involved in regenerating the pterin cofactor in two steps. 4**a**-Hydroxytetrabiopterin is first converted by 4**a**-carbinolamine dehydratase to quinonoid dihydrobiopterin, and the latter compound is then reduced back to tetrahydrobiopterin by NADH-dependent dihydropteridine reductase (Kaufman, 1987).

4a-Carbinolamine Dehydratase/DCoH

4**a**-Carbinolamine dehydratase was first purified from rat liver as a fraction called "phenylalanine hydroxylase stimulator", which could stimulate the hydroxylation reaction at pH 8.2 to 8.4 (Huang *et al.*, 1973). It was later found to be an enzyme that catalyzes the conversion of 4**a**-hydroxytetrabiopterin to the quinonoid dihydropterin (Lazarus *et al.*, 1983). 4**a**-Hydroxytetrabiopterin is also known to be unstable, breaking down nonenzymatically to the corresponding quinonoid dihydropterin (Kaufman, 1975). However, in the

absence of 4**a**-carbinolamine dehydratase, the dehydration of the 4**a**-carbinolamine becomes rate-limiting for the hydroxylation of phenylalanine. The consequent accumulation of 4**a**-carbinolamine results in a small percentage of rearrangement to the 7-tetrahydrobiopterin isomer (Curtius *et al.*, 1990). The latter 7-isomer was shown to be a potent inhibitor of the phenylalanine hydroxylase (Davis *et al.*, 1992). Under conditions where 4**a**-carbinolamine and the 7-isomer are generated, the addition of 4**a**-carbinolamine dehydratase markedly inhibits the rate of formation of the 7-isomer by diverting a greater fraction of the 4**a**-carbinolamine to the quinonoid dihydropterin (Davis *et al.*, 1991). Thus, the dehydratase not only directly catalyzes the dehydration of the carbinolamine, but also indirectly prevents isomerization to the inhibitory 7-isomer (Kaufman *et al.*, 1993).

4**a**-Carbinolamine dehydratase from rat liver has been cloned and sequenced (Citron *et al.*, 1992). It then became apparent that this dehydratase is identical to DCoH, a protein that facilitates the dimerization of hepatic nuclear factor 1 alpha (HNF-1α), a homeodomain transcription factor. DCoH was found to display a restricted tissue distribution and did not bind directly to DNA. The formation of a stable tetrameric DCoH-HNF-1α complex does not change the DNA-binding characteristics of HNF-1α, but does enhance the transcriptional activity of HNF-1α (Mendel *et al.*, 1991). X-ray crystallography has revealed DCoH to form a tetramer

containing two saddle-shaped grooves that comprise likely macromolecular binding sites (Endrizzi *et al.*, 1995). Structural similarities between the DCoH and nucleic acid-binding proteins imply that the saddle motif has evolved to bind diverse ligands or that DCoH may bind nucleic acid according to Endrizzi *et al.* (1995).

DCoH homologues have been identified in *Xenopus* (XDCoH) (Pogge-yon-Strandmann & Ryffel, 1995) and *P. aeruginosa* (PhhB) (Zhao *et al.*, 1994). XDCoH was found to be a maternal factor. The amount of XDCoH increases dramatically following neurulation, when the formation of liver, pronephros, and other organs takes place. The tissue distribution of XDCoH during embryogenesis suggests that XDCoH is involved in determination and differentiation of various unrelated cell types. The interaction with XDCoH was found to be essential for the function of several tissue-specific transcription factors (Pogge-yon-Strandman & Ryffel, 1995). In *P. aeruginosa* expression of *phhA* (encoding phenylalanine hydroxylase) was reported to require *phhB* (encoding 4**a**-carbinolamine dehydratase), suggesting that PhhB may have a positive regulatory role. If so, this would be an intriguing parallel with the dual catalytic and regulatory roles of the corresponding mammalian homolog (Zhao *et al.*, 1994).

Dihydropteridine Reductase

Dihydropteridine reductase (DHPR; EC 1.6.99.7) is one of the two essential enzymes involved in recycling the pterin cofactor for aromatic amino acid hydroxylases. It catalyzes the reduction of quinonoid dihydropterin to tetrahydrobiopterin, using NADH as a cofactor. DHPR is an ubiquitous enzyme in animals, being found in all tissues that contain the aromatic amino acid hydroxylases (Armarego *et al.*, 1984). Close correlation between levels of 4**a**-carbinolamine dehydratase and dihydropterine reductase in liver during human fetal development strongly suggests a physiologically significant role for both enzymes in tetrahydrobiopterin regeneration. Genetic defects in DHPR cause malignant phenylketonuria. A concomitant deficiency of neurotransmitters such as 3,4-dihydroxyphenylalanine (DOPA) and 5-hydroxytryptophan reflects the essential coupling of DHPR to tyrosine hydroxylase and tryptophan hydroxylase as well (Gudinchet *et al.*, 1992).

DHPR is also found in bacteria. DHPR has been purified from *Pseudomonas acidovorans* (Williams *et al.*, 1976) and *E. coli* (Vasudevan *et al.*, 1988). In *P. acidovorans*, both DHPR and phenylalanine hydroxylase activities were found to be higher in cells adapted to a medium containing *L*-phenylalanine or *L*-tyrosine as the sole carbon source than in those grown in *L*-asparagine (Williams *et al.*, 1976). Interestingly, DHPR has also been found in *E. coli* even though no aromatic amino acid

hydroxylases or 4**a**-carbinolamine dehydratase have ever been detected (Vasudevan *et al.*, 1988). Unlike other dihydropteridine reductases that have been studied, the *E. coli* DHPR possesses an FAD prosthetic group, and has dihydrofolate reductase and pterin-independent oxidoreductase activities (Vasudevan *et al.*, 1992).

Regulation of Phenylalanine Hydroxylase

Phenylalanine hydroxylase in mammals is tightly regulated at different levels. At the protein level, it is allosterically regulated by phenylalanine (Kaufman, 1987). The activity of phenylalanine hydroxylase increases at least 20-fold after incubation with phenylalanine (Tourian, 1971). It is also activated through phosphorylation by a cAMP-dependent kinase both *in vivo* and *in vitro* (Abita *et al*, 1976). At the DNA level, expression of the phenylalanine hydroxylase gene in liver and kidney tissues of mice is enhanced at birth and is induced by glucocorticoids and cAMP in liver (Faust *et al.*, 1996). Regulatory elements including a tissue-specific and hormone-inducible enhancer in the upstream region have been characterized. The enhancer region contains separate protein-binding sites for the glucocorticoid receptor and the hepatocyte-enriched transcription factor, hepatocyte nuclear factor 1 (HNF1) (Faust *et al.*, 1996). HNF1 is a transcriptional activator of many hepatic genes including albumin, α-antitrypsin, and α- or β-fibrinogen. Mice lacking

HNF1 die with a marked liver enlargement. The gene coding for phenylalanine hydroxylase is totally silent, thus giving rise to phenylketonuria (Pontoglio *et al.*, 1996).

Little information is available about the regulation of phenylalanine hydroxylase in bacteria. However, some evidence has indicated that the bacterial phenylalanine hydroxylase is also regulated. In *Pseudomonas acidovorans*, a higher level of phenylalanine hydroxylase was found after growth in the presence of phenylalanine (Willams *et al*, 1976). Induction of both phenylalanine hydroxylase and tryptophan hydroxylase in the presence of their substrates was also reported in *C. violaceum* (Letendre *et al*, 1974).

The most extensively characterized microbial phenylalanine system is that of *P. aeruginosa*. Whether this system is subject to any regulatory controls has not been studied prior to this work. The initial report of Zhao *et al.* (1994) provided a strong basis for anticipation that the phh operon would be subject to regulation for the following reasons. (i) The closely spaced organization of the three structural genes (*phhABC*) in an apparent operon implies regulation. (ii) Analysis of effects of the presence or absence of regions immediately flanking the phh operon upon expression of phenylalanine hydroxylase indicated the likely location there of one or more regulatory genes. (iii) The reported lack of *phhA* expression in the absence of *phhB*

suggested a positive regulatory role of *phhB* in addition to its catalytic function.

The major objectives of this study have been to elucidate the physiological conditions under which regulation occurs, to identify and characterize at the molecular-genetic level any regulatory genes which control the *phh* operon, and to determine the nature of the apparent positive regulatory action of *phhB*.

CHAPTER 2
PHHR, A DIVERGENTLY TRANSCRIBED ACTIVATOR OF THE PHENYLALANINE HYDROXYLASE GENE CLUSTER OF *Pseudomonas aeruginosa*

Introduction

A recent report (Zhao *et al.*, 1994) revealed that *Pseudomonas aeruginosa* possesses a tetrahydrobiopterin (BH_4)-dependent monooxygenase that is capable of catalyzing the phenylalanine hydroxylase reaction. It is encoded by the proximal member (*phhA*) of a three-gene cluster. The second gene, *phhB*, encodes carbinolamine dehydratase, a key enzyme within the cycle regenerating BH_4. *phhC* encodes an aromatic aminotransferase and belongs to the Family-I aminotransferases (Gu and Jensen, 1996). The reactions, as they are known to function for the mammalian homologs in the catabolism of *L*-phenylalanine, are shown in Fig. 2-1.

The physiological function of phenylalanine hydroxylase in *P. aeruginosa* has not been obvious. A primary role in *L*-tyrosine biosynthesis seems unlikely because of the established presence for this purpose of a cyclohexadienyl dehydrogenase that is widely distributed in gram-negative bacteria and which is highly sensitive to feedback inhibition by *L*-tyrosine (Xia and Jensen, 1990). Although function as an initial step of *L*-phenylalanine catabolism has precedent in

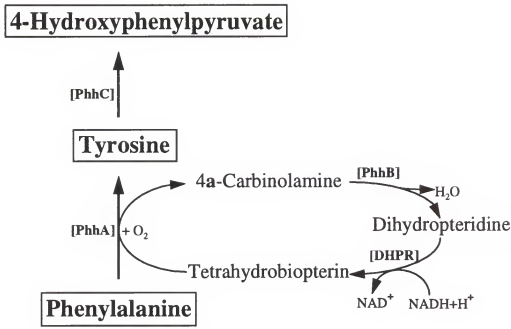


FIG. 2-1. Initial reactions of phenylalanine catabolism in mammals. The three structural genes of the *phh* operon encode enzymes catalyzing three of the four steps shown. The abbreviations: PhhA, phenylalanine hydroxylase; PhhB, 4**a**-carbinolamine dehydratase; PhhC, aromatic aminotransferase; DHPR, dihydropteridine reductase. 4**a**-Carbinolamine is an alternative designation for 4**a**-hydroxytetrahydrobiopterin.

mammalian metabolism, the literature encompassing the widely studied catabolism of aromatic compounds in pseudomonad bacteria (indeed, in prokaryotes) does not include the phenylalanine hydroxylase step. Furthermore, *L*-phenylalanine (substrate of PhhA) is an extremely poor source of carbon for growth of *P. aeruginosa*, whereas *L*-tyrosine (product of PhhA) is an excellent carbon source.

Zhao *et al.* (1994) had previously noted that subclones lacking the flanking regions around the *phh* operon possessed 20-fold greater activity for phenylalanine hydroxylase. This suggested the presence of a regulatory gene. Since an understanding of the regulation governing the *phh* operon should provide important physiological clues about function, I have analyzed the flanking regions and now report the characteristics of a regulatory gene, denoted *phhR*.

Materials and Methods

Materials

The bacterial strains and plasmids used in this study are listed in Table 1. The LB and M9 formulations (Sambrook *et al.*, 1989) were used as growth media for *E. coli* and *P. aeruginosa*. Pseudomonas isolation agar (Difco) was used for isolating *Pseudomonas* "knockout" mutants. Additions of ampicillin (100 μg/ml), chloramphenicol (40 μg/ml), kanamycin (50 μg/ml), tetracycline (25 μg/ml), mercuric chloride (15 μg/ml), *L*-phenylalanine (50 μg/ml), and thiamine (17 μg/ml)

TABLE 2-1. Bacterial strains and plasmids

Strain or plasmid	Relevant genotype or description	Source or reference
E. coli		
BL21(DE3)	F^- *ompT* $hsdS_B$ ($r_B^-m_B^-$) *gal dcm*; with DE3, a λ prophage carrying the T_7 RNA polymerase gene	Novagen
BW545	Δ*(lacU)169 rpsL*	Rosentel *et al.*
DH5α	F^-Δ*lacU169 φ80dlacZΔM15 hsdR17 recA1 endA1 gyrA96 thi-1 relA1 supE44*	GIBCO/BRL
LE392	F^-*e14*$^-$*(McrA*$^-$*)hsdR514*($r_k^-m_k^+$) *supE44 supF58 lacY1* or Δ*(lacIZY)6 galK galT22 metB1 trpR55*	Sambrook *et al.*
JP2255	*aroF363 pheA361 pheO352 tyrA382 thi-1 strR712 lacY1 xyl-15*	Baldwin & Davidson
S17-1	[RP4-2(Tc:Mu)(Km:Tn7)Tra(incP)] *pro hsdR recA* Tp^r Sm^r	Simon *et al.*
SP1312	*zah-735*:Tn*10* Δ*(argF-lac)U169*	Heatwole & Somerville
SP1312 (λSLW20)	SP1312 *φ(mtr'-lacZ*$^+$*)*	Heatwole & Somerville
SP1313	SP1312, Δ*(tyrR)*	Heatwole & Somerville
SP1313 (λSLW20)	SP1313 *φ(mtr'-lacZ*$^+$*)*	Heatwole & Somerville,
P. aeruginosa		
PA103	Prototroph	Totten *et al.*
PA103NG	*rpoN*	Totten *et al.*
PAO-1	Prototroph	Holloway
JS101	PAO-1 *phhA*, Hg^r	This study
JS102	PAO-1 *phhR*, Hg^r	This study

Table 2-1. (continued)

Plasmids		
pUC18	Amp^r lac'IPOZ'	Yanisch-Perron *et al.*
pUC19	Amp^r lac'IPOZ'	Yanisch-perron *et al.*
pACYC184	P15A replicon, Cm^r Tc^r	Chang & Cohen
pET11b	T7*lac* promoter, lacI$^+$ Ap^r	Novagen
pRS1274	*lacZY* fusion vector	Simons *et al.*
Z1918	Promoterless *lacZ*, Ap^r	Schweizer
pJZ9	*phhRABC*, Ap^r	Zhao *et al.*
pJZ9-3a	*phhAB*, Ap^r	Zhao *et al.*
pJS7	*phhRABC*, Ap^r	This study
pJS60	*phhABC*, Ap^r	This study
pJS61Z	*phhRA'-lacZ* transcriptional fusion, Ap^r	This study
pJS62Z	*phhA'-lacZ* transcriptional fusion, Ap^r	This study
pJS88	pET11b carrying *phhR* translational fusion at the ATG start site	This study
pJS91	pACYC184 carrying *phhR*$^+$, Cm^r	This study
pJS102	pRS1274 carrying *phhR'-lacZY* transcriptional fusion	This study
pCRII	Amp^r Kan^r lacZα	Invitrogen
pDG106	Hg^r Km^r P15A replicon	Gambill & Summers
pJS101	*Pst*I-*Sma*I fragment of pDG106 inserted into pUC18	This study
pUFR004	ColE1 Cm^r Mob$^+$ *mob*(P)	DeFeyter *et al.*

were made as appropriate. Agar was added at 20 g/liter for preparation of solid medium. Restriction enzymes, T4 DNA ligase, DNA-modifying enzymes (New England Biolab or Promaga) and *Taq* DNA polymerase (Perkin-Elmer) were used as recommended by the suppliers. Other biochemicals were purchased from Sigma Chemical Co. Inorganic chemicals (analytical grade) were from Fisher Scientific.

Phenylalanine Hydroxylase Assay

Cultures of *E. coli* JP2255 carrying the various plasmids specified were grown in 500 ml of LB broth supplemented with ampicillin (100 μg/ml) at 37°C and harvested at late exponential phase of growth. The cell pellets were resuspended in 10 ml of 10 m*M* potassium phosphate buffer (pH 7.4) containing 1 m*M* dithiothreitol and were disrupted by sonication for 30 s at 4°C using a Lab-Line Ultratip Labsonic System (Lab-Line Instruments, Inc., Melrose Park, IL). The resulting extracts were centrifuged at 150,000 x *g* for 1 hr at 4°C. The supernatant (crude extract) was desalted using Sephadex G-25 and used for enzyme assay. PhhA was assayed by following tyrosine formation (Nakata *et al.*, 1979).

Recombinant DNA Techniques

Molecular cloning and DNA manipulation, including plasmid purification, restriction enzyme digestion, ligation, and transformation were conducted by standard methods (Sambrook *et*

al., 1989). DNA fragments were purified from agarose gel with a "Geneclean" kit (Bio101). Electroporation (Invitrogen) was used for simultaneous transformation of *E. coli* with two compatible plasmids.

Construction of PhhR Expression Vectors

For expression of PhhR protein in *E. coli*, the T7 expression system (Novagen) was employed. The *phhR* coding region was cloned into a translational fusion vector pET11b. Polymerase chain reaction (PCR) was used to amplify the *phhR* gene. The upper primer (5'-ATACAT**ATG**CGTATCAAAGTGCACTGC-3') was made with a built-in *Nde*I restriction site (underlined) which allows fusion of *phhR* at the translational start site (ATG in bold). The lower primer (5'-CCTCCACCGTTTCTTTCCCAGCCT-3') was chosen at a position 48 bases downstream of the translational stop codon. PhhR protein made from this PCR fragment was designed to be a native protein, not a fusion protein. The PCR fragment was cloned into a TA cloning vector, pCRII. The *phhR* gene was excised from pCRII with *Nde*I and *Eco*RI. The *Nde*I-*Eco*RI fragment was first ligated with *Eco*RI-*Bam*HI adaptor to create a *Nde*I-*Bam*HI fragment which was then ligated with pET11b digested with *Nde*I and *Bam*HI to create the PhhR expression plasmid, pJS88 (Fig.2-3A).

For construction of a PhhR constitutive expression plasmid, pACYC184 was chosen as the expression vector. The pACYC184 vector has a P15A origin of replication which is

compatible with most commonly used plasmids using a ColEI origin of replication, and it has low-copy number (about 20 copies/cell). High level of PhhR produced from a high-copy number plasmid was found to be toxic to the host cells. The *Bgl*II-*Bam*HI fragment carrying *phhR* gene was excised from the expression plasmid pJS88 and cloned into the *Bam*HI site of pACYC184, thereby interrupting the tetracycline resistance gene (Tc^r) (Fig.2-3A).

Evaluation of Sensitivity/Resistance to m-*Fluoro-tyrosine*

Three *E. coli* strains, SP1312 (*tyrR*$^+$), SP1313 (*tyrR*$^-$) carrying pJS91 (*phhR*$^+$), and SP1313 (*tyrR*$^-$) carrying pACYC184 (*phhR*$^-$), were compared for sensitivity to *m*-fluoro-tyrosine (MFT). All three strains were first grown in M9 medium with appropriate antibiotics up to late-exponential phase of growth and then used to swab M9 agar plates containing appropriate antibiotics. A sterile Difco concentration disk (0.6 cm) was positioned at the center of each plate, and 10 μl of 50 μg/ml *m*-fluoro-tyrosine was applied onto the disks. The plates were then incubated at 37°C for 24 hours.

Construction of *phhA'-lacZ* and *phhR'-lacZ* Transcriptional Fusions

To compare levels of *phhA* transcription in both pJZ9 and pJZ9-3a, plasmids pJS61Z and pJS62Z were constructed, respectively. These have a promoterless *lacZ* gene (from

plasmid Z1918) fused at the *Bam*H I site within *phhA* to form *phhA'-lacZ* transcriptional fusions. Plasmid pJS61Z has the same upstream sequence as plasmid pJZ9, and plasmid pJS62Z has the same upstream sequence as pJZ9-3a. Hence, the *phhA'-lacZ* fusions in pJS61Z and pJS62Z should represent the *phhA* transcriptional levels in pJZ9 and pJZ9-3a, respectively.

To study regulation of the *phhR* promoter, the *Hinc*II-*Bam*HI fragment (*phhR'*) was cloned into the pRS1274 *lacZY* transcriptional fusion vector at the *Bam*HI-*Sma*I site to create pJS102(*phhR'-lacZ*).

ß-Galactosidase Assay

ß-Galactosidase activity was assayed under conditions of proportionality as described by Miller (1972), and specific activities are expressed in Miller units. The data are the results of at least two independent assays.

Gene Inactivation

P. aeruginosa is well known for its relatively high resistance to most antibiotics, which complicates attempts to use most of available antibiotic-resistance genes as selective markers for gene replacement. Mercury resistance (Hg^r) was used as a selective marker since *P. aeruginosa* has been shown to be sensitive to mercury (Essar *et al.*, 1990; Gambill and Summers, 1985). Insertional inactivation technique described by Sophien *et al.* (1992) utilizes a mobilizable suicide vector

containing a truncated gene fragment (at both 5' and 3' ends) and Hg^r-cassette, and this suicide plasmid was integrated into the chromosome by a single homologous recombination event. PCR was used to generate truncated fragments. To generate a '*phhR*' (601bp) fragment, the upper primer 5'-CCGTGTAGGCATCCTCCGCGACAT-3', and the lower primer 5'-CTGGAAGATACTGTCGAAGCCACG-3' were used; to generate the '*phhA*' (639bp) fragment, we used the upper primer 5'-ACGACAACGGTTTCATCCACTATC-3' and the lower primer 5'-GGACGAAATAGAGCGGTTGCAGGA-3'. The PCR-generated fragments were cloned into pCRII (a TA cloning vector) and then excised with *Eco*RI. The *Eco*RI fragments were subsequently cloned into the *Eco*RI site of pUFR004 (a mobilizable suicide vector) to create pUFR/'*phhA*', and Hg^r *Hin*dIII-cassette from pJS101 was inserted into the *Hin*dIII site of pUFR/'*phhA*' to create pUFR/'*phhA*'/Hg^r. pUFR/'*phhR*'/Hg^r was created in a similar fashion. These plasmids were then used to transform *E. coli* strain S17-1 (a mobilizing strain). Strain S17-1 harboring either pUFR/'*phhA*'/Hg^r or pUFR/'*phhR*'/Hg^r was used as the donor in biparental mating with *P. aeruginosa* performed as described by Simon *et al.* (1983). Donor and recipient cells were grown in LB broth to an OD_{600} of about 1.0 (*E. coli* S17-1 at 37°C and *P. aeruginosa* PAO-1 at 42°C), mixed (0.5 ml volume of each) in a 1.5-ml microcentrifuge tube, and pelleted by centrifugation. The mating mixture was carefully resuspended in 0.2 ml of LB broth and spread onto a sterile nitrocellulose filter (0.45-μm

pore size) resting on a prewarmed LB agar plate. The plates were incubated for 16-24 hours at 37°C, and then cells were removed from the filter by an inoculation loop and resuspended by vortexing into 0.5 ml of LB broth. Aliquots of 10-, 20-, 50-, and 100-μl volume of the cell suspension were spread onto Pseudomonas isolation agar plates containing 15 μg of $HgCl_2$. The plates were incubated overnight and Hg^r colonies were isolated.

Preparation of PhhA-specific Polyclonal Antiserum

PhhA was partially purified by anion-exchange and gel-filtration chromatography following the methods described by Zhao *et al.*, (1994). The partially purified PhhA was subject to SDS-PAGE (12%) and the gel was stained with Commassie blue R-250. The PhhA band was cut from the gel and used for the production of polyclonal antiserum in rabbits (Cocalico Biologicals, Inc., Reamstown, PA). Antiserum was purified by using an Econo-Pac protein A column (Bio Rad) and further absorbed with a total cell extract from the PhhA-deficient mutant JS101.

SDS-PAGE and Western Blot Analysis

SDS-PAGE (12%) was performed with the Mini-PROTEAN II Cell (Bio-Rad) by the method of Laemmli (1970). Samples of exponential-phase cells were collected by centrifugation, and the cell pellets were suspended in gel-loading buffer and

heated at 100°C for 10 min. Samples of 5-10 μl were loaded onto two SDS-acrylamide gels. After separation of the proteins by electrophoresis, one gel was stained with Coomassie blue R-250 and the other gel was used for blotting. When crude extracts were used, equivalent amounts of protein were loaded in each lane. Western blots were performed according to Towbin *et al.* (1979). The proteins were eletrophoretically transferred onto nitrocellulose membranes and reacted with polyclonal antibodies raised against PhhA in a rabbit.

N-Terminal Amino Acid Sequencing

PhhR protein produced in *E. coli* BL21(DE3)/pJS88 following induction by 1 m*M* IPTG for 3 hours was first separated from the whole lysate by SDS-PAGE. The proteins were then blotted to a polyvinylidene difluoride membrane (Bio-Rad) and were stained with Coomassie brilliant blue R-250 (Sigma). The band corresponding to PhhR was excised from the membrane and used for sequencing by using an Applied Biosystems model 407A protein sequencer with an on-line 120A phenylthiohydantoin analyzer in the Protein Core Facility of the ICBR at the University of Florida.

DNA Sequencing and Data Analysis

Sequencing of *phhR* region was performed by the DNA Sequencing Core Laboratory of the University of Florida. The nucleotide sequence and the deduced amino acid sequence were

analyzed by using the updated version of sequence analysis software package offered by the Genetics Computer Group (GCG) of University of Wisconsin (Devereux *et al.*, 1984).

Nucleotide Sequence Accession Number

The nucleotide sequence reported in this work has been assigned Genbank accession number U62581.

Results

Evidence for a Flanking Regulatory Region

The original clone (pJZ9) isolated by Zhao *et al.* (1994) produced markedly less phenylalanine hydroxylase activity than did subclone pJZ9-3a (Fig. 2-2A). A possible explanation was the presence of a negatively-acting regulatory gene in either the upstream or downstream flanking region. Plasmid pJS60, in correlation with its absence of upstream DNA but presence of downstream DNA, expressed a very high level of activity. Thus, the upstream region appeared to be responsible for decreased expression of *phhA* in *E. coli*.

Transcriptional fusions were constructed using *lacZ* as a reporter gene, as diagrammed in Fig. 2-2B. The results indicate that the negative effect conferred by upstream DNA occurs at the transcriptional level.

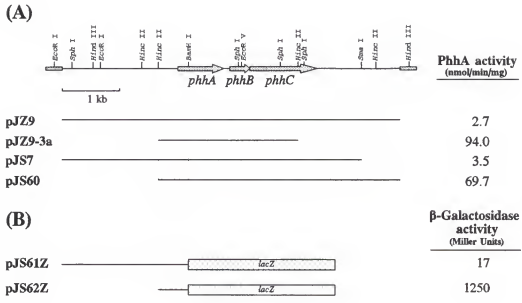


FIG. 2-2. Localization of a regulatory region upstream of the *phh* operon. (A) On the right phenylalanine hydroxylase (PhhA) activities are shown in *E. coli* JP2255 harboring different plasmids shown on the left. (B) On the right β-galactosidase activities are shown in BW545 harboring the *phhA'-lacZ* transcriptional fusions diagrammed on the left.

Identification of *phhR*

A large open reading frame (Fig. 2-3) located upstream of the *phh* structural genes appeared likely to be functional on the criterion of GCG codon preference analysis. The gene, denoted *phhR*, produces a deduced protein having 518 residues, an anhydrous molecular weight of 56,855, and an isoelectric point of 7.17. It contains a single tryptophan residue.

Regions corresponding to a possible σ^{70} promoter region and a factor-independent transcription terminator are marked in Fig.2-3. A strong ribosome-binding site was not apparent. Bases that are complementary to *P. aeruginosa* 16S rRNA at the 3' terminus are marked. Perhaps the "A-richness" of the initiator region enhances ribosome binding (Ivey-Hoyle and Steege, 1992).

A physical map is given in Fig. 2-4 of the 5874-bp DNA segment containing the structural genes of the *phh* operon, the divergently transcribed regulatory gene *phhR*, and a gene (*pbpG*) downstream of the *phh* operon which encodes a penicillin-binding protein (Song and Jensen, unpublished data).

Homology of PhhR with E. coli TyrR

The closest homolog of PhhR was found to be *E. coli* TyrR. The pairwise GAP alignment (GCG) is shown in Fig. 2-5. TyrR belongs to a family of modular proteins which usually have three functional domains. The alignment showed high level of

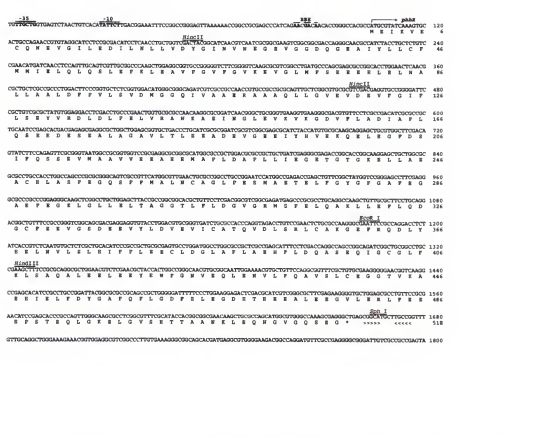
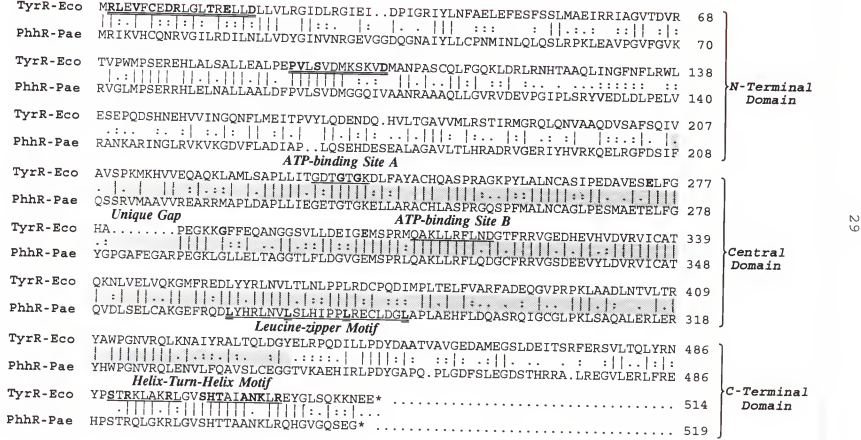


FIG. 2-3. Nucleotide sequence of the *phhR* region. The numbers at the right indicate nucleotide and amino acid positions. The putative promoter region and ribosome-binding site (RBS) are indicated with bold print. RBS bases that are complementary to *P. aeruginosa* 16S rRNA are overlined. The translational start site is indicated by a bent arrow and the stop codon by an asterisk. Nucleotides forming the complementary stems of the putative transcriptional terminator are marked with tandem arrowheads. Restriction endonuclease recognition sites are marked above the nucleotide sequence.

FIG. 2-4. Physical map of the DNA fragment containing *phh* structural genes, the divergently-transcribed regulatory gene *phhR*, and the downstream penicillin-binding protein gene (*pbpG*) in pJZ9. Terminators downstream of *phhC* and *phhR* are indicated. The shaded bars at both ends are portions of the multiple cloning site of the pUC18 vector. The location of restriction sites is shown.

FIG. 2-5. Pairwise alignment (GAP program of GCG) of amino acid sequences corresponding to *E. coli* TyrR and *P. aeruginosa* PhhR. The similarity is 66.3% and identity is 45.7%. The three functional domains are indicated at the right, the central domain also being shaded. Domain boundaries are based upon those formulated by Morrett and Segovia (1993). Alernative boundaries based upon domain segments surviving partial hydrolysis by trypsin (Cui and Somerville, 1993) are residues 1-190, 191-467, and 468-513 for the three domains of Eco-TyrR, respectively. In the N-terminal domain, the region between amino acids 2 and 19 that have a critical role in activation of the expression of *E. coli tyrP* and *mtr* (Pittard, 1996) is double-underlined, and mutations at residues marked in boldface type abolish TyrR-mediated activation without affecting repression; a second region between amino acids 92 and 103 which may play a subsidiary role in activation is also double-underlined; mutations at the residues in boldface type resulted in loss of function (Pittard, 1996). In the central domain, two ATP-binding sites and a leucine-zipper motif are underlined. Mutations altering ATP-binding site A and mutations at the highly conserved residues E-274, G-285, and E-302 abolish TyrR-mediated repression. In the C-terminal domain, a helix-turn-helix DNA-binding motif is identified with the helix regions underlined and critical residues in bold print.


TyrR-Eco
PhhR-Pae
N-Terminal Domain
ATP-binding Site A
Unique Gap
ATP-binding Site B
Central Domain
Leucine-zipper Motif
Helix-Turn-Helix Motif
C-Terminal Domain

conservation throughout the entire length of PhhR and TyrR, and 45.7% of the deduced residues were identical. The N-terminal domain mediates regulatory modulations, and in TyrR it binds all three aromatic amino acids. A central domain, highly conserved throughout the entire family of σ^{54} enhancer-binding proteins, exhibits two established motifs that reflect the binding of ATP (Pittard, 1996). Site A corresponds to the ATP-binding pocket motif and site B corresponds to segment 3 of adenylate kinase. In this region a perfect leucine-zipper motif is apparent in *P. aeruginosa* PhhR, whereas *E. coli* displays an imperfect motif. Residues E-274, G-285, and E-302 were found to be important for TyrR-mediated repression of *aroF-tyrA* in *E. coli* (Yang *et al.*, 1993; Kwok *et al.*, 1995), and these residues are all conserved in *P. aeruginosa* PhhR. The C-terminal domain possesses a helix-turn-helix motif which is responsible for DNA binding. The absolute conservation of residues shown to be critical in *E. coli* (Pittard, 1996) strongly indicates that PhhR and TyrR might target to a similar DNA sequences.

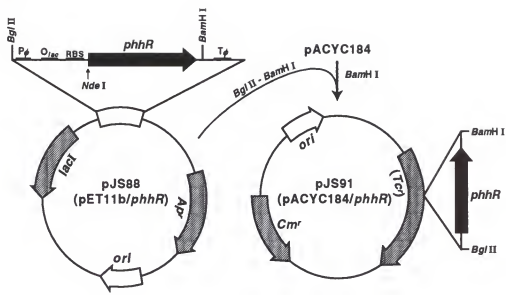
Similar to *E. coli* TyrR, the two aspartate residues and the lysine residue conserved in the amino-terminal domain of all response regulator proteins (Stock *et al.*, 1989) were not found.

Overproduction of PhhR

PhhR protein was overexpressed in *E. coli* BL21(DE3) as detailed under *Materials and methods* by use of the T7 overexpression system; the construct is illustrated in Fig.2-6. The initial use of overexpression vectors containing *phhR* on the *Bam*HI-*Sph*I fragment (see Fig. 2-4) of pJZ9 failed. This is probably due to autogenous regulation of *phhR*, judging from the precedent set by *tyrR* in *E. coli* (Argaet *et al.*, 1994). Accordingly, overexpression was achieved through excision of DNA upstream of *phhR*. PCR methodology was used to generate an intact *phhR* gene which was fused with the T7 translational start codon at a *Nde*I restriction site to create overexpression plasmid, pJS88. *E. coli* BL21(DE3) that had been transformed with pJS88 was induced with 1 mM IPTG for 3 hours to express PhhR. Whole-cell lysates obtained before and after IPTG induction were analyzed by SDS-PAGE, as shown in Fig. 2-6B. Overproduction of a 56-kDa protein was observed, and N-terminal amino acid sequencing confirmed its synonymy with PhhR.

Initial attempts to express *phhR* in *E. coli* under physiological conditions indicated that expression of *phhR* is highly toxic. The *Bgl*II-*Eco*RI fragment from pJS88 was cloned into the *Bam*HI-*Eco*RI site of pUC19 behind a *lac* promoter. When transformed into *E. coli* DH5α, transformants achieved only pinpoint colony size and eventual survivor cells inevitably had lost the plasmid. Success was finally achieved by use of

(A)



(B)

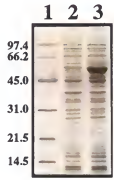


FIG. 2-6. Overproduction of PhhR protein. (A) Map of PhhR overexpression plasmid pJS88 (left), and low-copy number, constitutive PhhR expression plasmid pJS91 (right). (B) SDS-PAGE analysis of whole-cell lysate of *E. coli* BL21(DE3) harboring pJS88. The gel was stained with Coomassie blue. Lane 1, molecular-weight markers; lane 2, before IPTG induction; lane 3, induced by 1 m*M* IPTG for 3 h.

pACYC184, a low copy-number plasmid, to create pJS91 which carried the *Bgl*II-*Bam*HI fragment of pJS88 ligated into the *Bam*HI site of pACYC184 (Fig. 2-6A). Analysis of 11 plasmids isolated showed that the orientation of *phhR* in each case was opposite to that of the Tc^r gene. Presumably, the higher level of expression expected when driven by the Tc^r promoter still confers an intolerable level of toxicity.

Functional Replacement of *E. coli* tyrR with phhR

A simple test was used to see whether *phhR* could substitute for *tyrR* as a repressor of the *aroF-tyrA* operon. Mutants deficient in TyrR exhibit resistance to *m*-fluorotyrosine (Fig. 2-7, middle) whereas *tyrR*$^+$ strains exhibit sensitivity to growth inhibitory effects of the analog (Fig. 2-7, left). pJS91 (*phhR*$^+$) was used to transform an *E. coli tyrR*-deficient background (strain SP1313). The ability of PhhR to replace TyrR is qualitatively apparent (Fig. 2-7, right) by inspection of the halo of growth inhibition on a bacterial lawn surrounding a disc containing *m*-fluorotyrosine in SP1313 (*tyrR*$^-$ *phhR*$^+$).

We also examined the ability of PhhR to replace TyrR as an activator of *mtr*, encoding a component of a tryptophan-specific transport system. The *phhR*$^+$ plasmid pJS91 was transformed into two *E. coli* λ lysogens (Heatwole and Somerville, 1991) which carried *mtr'-lacZ* transcriptional fusions integrated in the chromosome as single-copy fusions.

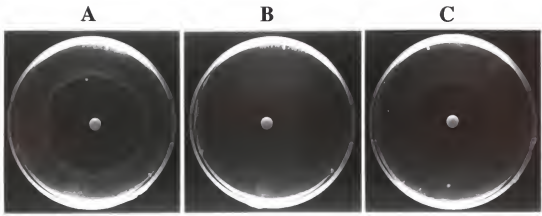


FIG. 2-7. Functional replacement of TyrR by *P. aeruginosa* PhhR in *E. coli*, as monitored by sensitivity to growth inhibition by *m*-fluoro-tyrosine (MFT). (A) *E. coli tyrR*$^+$ (wildtype) strain SP1312 is very sensitive to MFT present on a central disc, exhibiting a large zone of growth inhibition; (B) *E. coli tyrR*$^-$ strain SP1313 is insensitive to MFT, showing no zone of growth inhibition; (C) *P. aeruginosa phhR*(pJS91) *in trans* complements *E. coli tyrR*$^-$ and restores the sensitivity to MFT, as visualized by a zone of growth inhibition.

Strain SP1312 (*tyrR*$^+$) exhibited the expected elevation of ß-galactosidase activity following growth in the presence of tyrosine, phenylalanine, or both. However, strain SP1313 (*tyrR*$^-$) carrying pJS91 (*phhR*$^+$) produced the control level of ß-galactosidase activity, regardless of the presence or absence of aromatic amino acids (data not shown). Thus, PhhR appears to be incapable of replacing TyrR as an activator of *E. coli mtr*.

Autogenous Regulation of *phhR*

The *Bam*HI-*Hin*cII fragment containing the 5′ coding regions of *phhA* and *phhR* and the intervening region (see Fig. 2-4) was fused to *lacZ* to give the reporter-gene construct pJS102 (*phhR′-lacZ*). This plasmid construct was introduced into the *tyrR*-negative background of strain SP1313 in the presence or absence of pACYC184 possessing a *phhR*$^+$ insert. The results (Table 2-2) demonstrated a repressive effect of *phhR*$^+$ upon PhhR levels as monitored by measurement of *β*-galactosidase activity. Since the copy number of pJS91 (the source of PhhR molecules) in this experiment is lower than the number of repressor target sites provided by the high-copy number pJS102 and since TyrR boxes are present within seven other transcriptional units of *E. coli*, auto-regulation is undoubtedly grossly under-estimated due to titration of available PhhR molecules in the system.

Table 2-2. Autoregulation of *P. aeruginosa phhR* in *E. coli* SP1313(*tyrR*⁻) containing pJS102(*phhR'-lacZ*)

Second plasmid[a]	β-Galactosidase levels[b] in cells grown in: M9[c]	M9 + F	M9 + Y
pACYC184	550	510	589
pACYC184(*phhR*⁺)	362	376	372

[a] pACYC184(*phhR*⁺) is denoted pJS91 in Table 1.
[b] β-Galactosidase levels are reported in Miller Units.
[c] M9 minimal medium was supplemented with 1 m*M* thiamine-HCl and, where indicated, 1 m*M* phenylalanine (F), or 1 m*M* tyrosine (Y).

PhhR as A Positive Regulator

PhhR and TyrR form a cluster within the larger family of σ^{54} enhancer-binding proteins, as illustrated by Fig. 2-8. A *rpoN* mutant of *P. aeruginosa* was assayed by Western analysis for PhhA levels of expression in order to determine whether expression of the *phh* operon is dependent upon σ^{54} like most family members, or whether it is σ^{54}-independent like *tyrR* and *luxO*. Only low basal levels of PhhA were present in the *rpoN* mutant, indicating expression to be largely σ^{54}-dependent. This, in turn, implied that *phhR* might function as an activator protein for *phhABC* transcription. *phhR* was inactivated as described under *Materials and Methods*, and Western analysis of the effect upon PhhA level was carried out. The results (Fig. 2-9) indicated that *phhR* encodes an activator, the absence of which allows only a low basal level of activity.

The small molecules, *L*-phenylalanine and *L*-tyrosine, was found to function as an inducer (Fig. 2-10). Western analysis of PhhA showed no detectable band in minimal medium and a barely detectable band when *L*-tryptophan was present, compared to prominent bands when *L*-phenylalanine or *L*-tyrosine was additionally present. Carbon-source levels of *L*-phenylalanine or *L*-tyrosine were not required for induction. It is probable that *L*-phenylalanine or *L*-tyrosine is a co-activator moiety which, in combination with PhhR, forms the holo-activator moiety. It is perhaps relevant that for those transcriptional

FIG. 2-8. Homology relationships of the central domain of *P. aeruginosa* PhhR with the central domain of other members of the σ^{54}-dependent family of transcriptional regulators. The dendrogram was generated with amino acid sequences of the central domain as defined by Morrett and Segovia (1993) by using the PILEUP program of GCG. The top three proteins form a cluster designated as subfamily α, and the remaining proteins form a larger cluster designated as subfamily β. Due to their high degree of similarity, only one of the ortholog sequences of NifA, NtrC and HydG proteins is shown. The six paralogs from *E. coli* and the three paralogs from *P. aeruginosa* are designated with * and ♣, respectively. Abbreviations: Eco, *Escherichia coli*; Avi, *Azotobacter vinelandii*; Hin, *Haemophilus influenzae*; Pae, *Pseudomonas aeruginosa*; Vha, *Vibrio harveyi*. Functions controlled by the following regulators are given parenthetically: PhhR (phenylalanine hydroxylase), TyrR (aromatic amino acid biosynthesis and transport), VnfA (nitrogen fixation, nitrogenase-2), Anf (nitrogen fixation, nitrogenase-3), NifA (nitrogen fixation, nitrogenase-1), HydG (hydrogen oxidation), NtrC (nitrogen assimilation), PilR (synthesis of Type IV pili), AlgB (alginate production), LuxO (luminescence), FhlA (formate metabolism), YfhA (possible control of *glnB*), PspF (phage shock protein).

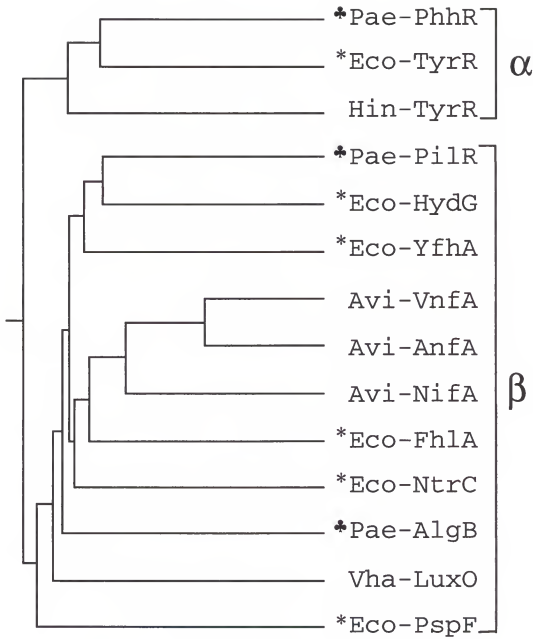

♣Pae-PhhR
*Eco-TyrR
Hin-TyrR
α
♣Pae-PilR
*Eco-HydG
*Eco-YfhA
Avi-VnfA
Avi-AnfA
Avi-NifA
*Eco-FhlA
*Eco-NtrC
♣Pae-AlgB
Vha-LuxO
*Eco-PspF
β

FIG. 2-9. Western blot analysis of *phhA* expression in mutant derivatives of *P. aeruginosa* strains PAO-1 and PA103. The proteins in crude extracts prepared from cultures grown in LB medium were separated by SDS-PAGE, and equal amounts of protein (50μg) were applied to each lane.

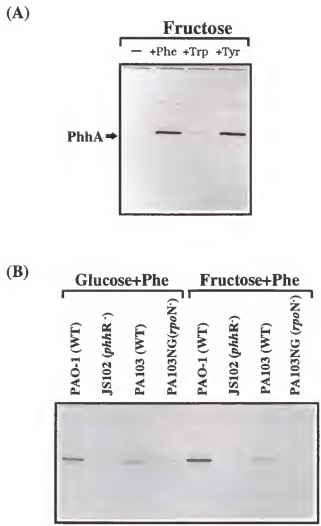


FIG. 2-10. Western blot analysis of *phhA* expression. (A) Examination of aromatic amino acids as inducers of *phhA* expression. *P. aeruginosa* PAO-1 was grown in minimal salts-glucose or minimal salts-fructose medium with or without addition of one of the three aromatic amino acids at a final concentration of 100 μg/ml. (B) Phenylalanine induction of *phhA* expression in different *P. aeruginosa* strains. Bacteria were grown in minimal-glucose or minimal-fructose media containing 100 μg/ml phenylalanine.

units where TyrR functions as an activator, *L*-phenylalanine functions as an essential co-activator (Pittard and Davidson, 1991).

Discussion

Anomalous Repression of the *phh* Operon by PhhR in *E. coli*

PhhR can mimic the ability of TyrR to repress the *aroF-tyrA* operon at a σ^{70} promoter. This indicates that PhhR can recognize TyrR boxes and is consistent with the high similarity of the helix-turn-helix, DNA-binding domain within the carboxy-terminal segments of TyrR and PhhR. However, PhhR was unable to activate the *phh* operon in the heterologous *E. coli* background, suggesting an incompatibility between the *E. coli* RpoN and the *P. aeruginosa* σ^{54}-dependent system. The expression of PhhA from a promoter recognized by *E. coli* upstream of the native σ^{54} promoter was in fact severely depressed in constructs containing *phhR*, even in the presence of added co-activator (*L*-phenylalanine). In the presence of *P. aeruginosa* PhhR, an aberrant complex apparently blocks transcription initiated upstream of the σ^{54} promoter.

Emerging Subfamilies within the σ^{54} Enhancer-Binding Protein Family

P. aeruginosa PhhR belongs to an outlying subgroup (which we denote subfamily α in Fig. 2-8) of the σ^{54} enhancer-binding protein family. All members of the family possess in common a

homologous central domain, but the amino-terminal and carboxy-terminal domains may vary considerably within the family. Thus, this exemplifies a complex multi-domain protein family in which family membership is defined by a common ancestral central domain. Future subdivisions within what is termed subfamily β in Fig. 2-8 could likely be defined on the criterion of homology for the remaining two domains. For example, Eco-NtrC and Eco-FhlA belong to different mechanistic subgroups: the two-component regulatory system and direct response-to-small-molecules, respectively (reviewed by Shingler, 1996).

Figure 2-8 highlights the emerging homology relationships of selected paralog and ortholog proteins, with respect to the central domain. *E. coli* possesses at least six paralogs, some of which diverged in a common ancestor that existed prior to speciation events which generated orthologs. Thus, the divergence of Eco-NtrC and Pae-PilR was a more recent event than was the divergence of Eco-NtrC and Eco-PspF. In contrast to the ancient duplication events which generated all of the *E. coli* paralogs (or the *P. aeruginosa* paralogs) are the relatively recent duplication events generating the three paralogs which regulate three distinctly separate nitrogenase systems in *Azotobacter* (Joerger *et al.*, 1989).

P. aeruginosa PhhR and *E. coli* TyrR exhibit homology in all three domains: 36% identity, amino-terminal; 52% identity, central; and 47% identity, carboxy-terminal). Curiously, the

amino-terminal domain of *H. influenzae TyrR* appears to be absent. It is not known whether sequencing errors might account for this, or whether the equivalent of the amino-terminal domain might exist separately as a different protein.

A multiple alignment of the central-domain modules of subfamilies α and β was shown in Fig. 2-11. In addition to the many residues that are absolutely conserved throughout the family, some residues which may prove to be uniquely conserved within subfamily α are apparent, e.g., APLL corresponding to residues 29-32 of Hin-TyrR.

Both Eco-TyrR and Rca-NtrC exhibit deletions in the "unique-gap region" of the central domain (Fig. 2-12) in correlation with their regulation of σ^{70} promoters, rather than σ^{54} promoters. This observation led to the suggestion (Morrett and Segovia, 1993) that this region of the central domain might be critical for functional interfacing with σ^{54}. Since this DNA segment of Pae-PhhR is intact with absolute retention of highly conserved residues, the foregoing hypothesis is consistent with the successful interaction of PhhR with a σ^{54} promoter. Hin TyrR, on the other hand, is likely to be deficient in interaction with σ^{54} (like *E. coli* TyrR), owing to a 6-residue deletion in this region.

Intervening Region of Divergent Transcription

Since the DNA-binding region of the carboxy terminus of PhhR is identical at all important residues with *E. coli* TyrR,

FIG. 2-11. A comparison of the amino acid sequences in the central domain of the PhhR protein and 13 other homologs. The sequences were aligned by using the PILEUP program of GCG. The numbering of amino acid residues is given on the left. Percent identity of PhhR with its homologs is given at the lower right. Amino acid residues conserved in all 14 sequences are in double-lined boxes. Amino acid residues conserved in 13 of 14 sequences are shaded. Conserved residues which are confined to either the top cluster (subfamily α) or the bottom cluster (subfamily β) are in single-lined boxes. Two ATP-binding motifs are indicated above the consensus sequences in boldface type. See the legend for Fig. 8 for abbreviations.

ATP-binding Motif A
ATP-binding Motif B

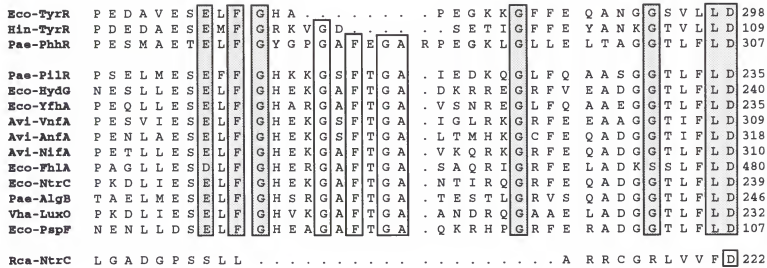


FIG. 2-12. Alignment of the unique-gap region in the central domains of TyrR proteins with selected homologs. Amino acid residues conserved in all of the 15 sequences that include both subfamily α (top cluster) and subfamily β (lower cluster) are in shaded boxes. Amino acid residues conserved within the gap region are shown in open boxes. Rca, *Rhodobacter capsulatus*; see the legend of Fig. 8 for other abbreviations used.

it is likely that PhhR binds to the same binding sites for *E. coli*, which are referred as "TyrR boxes" (consensus: TGTAAAN$_6$TTTACA). This conclusion is also supported by the ability of PhhR to replace TyrR as a repressor of the *aroF-tyrA* transcriptional unit. The location of two "PhhR boxes" which match the consensus for "TyrR boxes" was shown in Fig. 2-13. PhhR Box 1 is a strong box (with more conserved-symmetry and higher affinity for TyrR) that overlaps the putative -10 region of the *phhR* promoter. TyrR boxes in *E. coli* occur in tandem with variable spacing (Pittard, 1996), and a TyrR hexameric molecule is thought to bind both a strong box and a weak box with DNA looping in between. PhhR Box 2 is a weak box located in the middle of the intervening region. It seems probable that by analogy with autorepression of *tyrR* in *E. coli*, both *phhR* boxes participate in the autogenous repression of *phhR* by PhhR, probably with tyrosine as a corepressor.

In the opposite direction of transcription, the σ^{54} promoter for *phhABC* requires an upstream activator site (UAS). PhhR Box 1 may be the most likely UAS, although perhaps both boxes participate in activation of *phhA*. *L*-Phenylalanine and *L*-tyrosine, potent inducers of phenylalanine hydroxylase, presumably are the effector molecules. Since a *rpoN* mutant retained low basal level of PhhA, another promoter that is independent of σ^{54} might be present.

No motif for binding of integration host factor (IHF) (Friedman, 1988) was located in the intervening region.

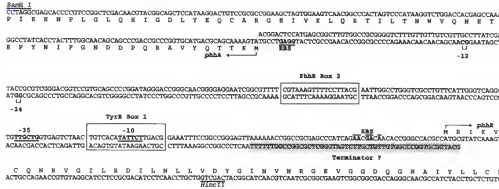


FIG. 2-13. The intervening sequence between the divergently transcribed *phhA* and *phhR* genes. The number at the end of each line indicates the nucleotide position. The ribosome binding sites and putative promoter sites (-12/-24 promoter for *phhA*, and -10/-35 promoter for *phhR*) are indicated. The translational start sites are indicated by arrows. Two PhhR boxes are identified. A stem-loop structure is shaded. Restriction endonuclease recognition sites are marked.

Therefore, this region may possess intrinsic DNA-bending capabilities.

Function of the *phh* Operon

The primary function of the *phh* operon is clearly not to accommodate tyrosine biosynthesis since the feedback-inhibited cyclohexadienyl dehydrogenase which is widely distributed in gram-negative bacteria exists for this purpose. However, the *phh* operon probably provides a fortuitous backup capability for tyrosine biosynthesis. "Reluctant auxotrophy" for tyrosine (Patel *et al.*, 1978) can be explained as follows. Mutational deficiency of cyclohexadienyl dehydrogenase would lead to accumulation of prephenate, a potent product inhibitor of chorismate mutase. The subsequent backup of chorismate, enhanced by lack of early-pathway control in the absence of *L*-tyrosine, results in passage of chorismate to the periplasm where chorismate mutase-F (Gu and Jensen, unpublished data) and cyclohexadienyl dehydratase (Zhao *et al.*, 1993) generate *L*-phenylalanine. Subsequent induction of phenylalanine hydroxylase completes the alternative circuit to *L*-tyrosine.

The established function of phenylalanine hydroxylase in mammals is for catabolism of *L*-phenylalanine as a carbon source. We have found that phenylalanine hydroxylase is indeed essential for use of *L*-phenylalanine as a sole carbon source in *P. aeruginosa*. Thus, inactivation of *phhA* resulted in inability to use *L*-phenylalanine as a sole source of carbon

(data not shown). However, induction of the *phh* operon under conditions where better carbon sources (such as glucose) coexist, suggests that the *phh* operon might be dedicated to provision of some specialized compound from *L*-phenylalanine.

Inactivation of *phhR* resulted not only in the inability to use *L*-phenylalanine as a carbon source, but also in an inability to use *L*-tyrosine as a carbon source. Since TyrR regulates aromatic amino acid permeases in *E. coli*, we considered the possibility that the *phhR* mutant might fail to grow on *L*-tyrosine because of a permease deficiency. Since MFT is likely to be transported by the same system as *L*-tyrosine, a permease-deficient phenotype should be resistance to growth inhibition by MFT. However, the phhR$^-$ mutant has a MFT-sensitive phenotype on fructase-based medium (data not shown). Therefore, PhhR might regulate steps of tyrosine catabolism.

Regulation of Multiple Transcriptional Units by PhhR?

TyrR represses or activates eight transcriptional units in *E. coli* (Pittard, 1996). Similarly organized transcriptional units are absent or unknown in *P. aeruginosa*. However, the counterpart of the *aroF-tyrA* operon in *P. aeruginosa* would be genes encoding tyrosine-sensitive DAHP synthase and cyclohexadienyl dehydrogenase. Physiological manipulations in our laboratory have never revealed repression control of these apparently constitutive enzymes. Consistent with this, PhhR exhibits no regulatory control of either of

these enzymes, on the criterion of assessment of specific activities determined in comparison of *tyrR*$^+$ and *tyrR*$^-$ backgrounds (data not shown).

CHAPTER 3
BIFUNCTIONAL PhhB REGULATES THE EXPRESSION OF PHENYLALANINE HYDROXYLASE IN *Pseudomonas aeruginosa*

Introduction

Mammalian 4**a**-carbinolamine dehydratase was initially known for its catalytic activity of converting 4a-carbinolamine to quinonoid dihydrobiopterin in regenerating the tetrahydrobiopterin for phenylalanine hydroxylase (Fig. 3-1B). Later, it was found to be synonymous with DCoH, the dimerization cofactor for hepatic nuclear factor 1 alpha (HNF-1α) (Citron *et al.*, 1992).

A homolog of the mammalian DCoH, PhhB, was found in *Pseudomonas aeruginosa* by Zhao *et al.* (1994). The PhhB protein is encoded by the second structural gene, *phhB*, of the *phh* operon (Fig. 3-1A). Zhao *et al.* (1994) reported that *phhB* is required for the expression of phenylalanine hydroxylase, encoded by the first structural gene, *phhA*. In the absence of the *phhB* gene, *phhA* by itself not only failed to complement *E. coli* tyrosine auxotrophy, but was not expressed in *E. coli* as indicated by SDS-PAGE. Dual catalytic and regulatory roles of PhhB are an intriguing possibility in the context of the fact that DCoH, the mammalian counterpart of PhhB, is a bifunctional protein with enzymatic activity as 4**a**-

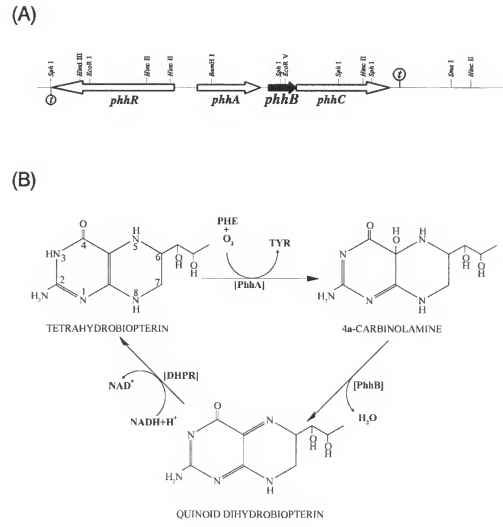


FIG. 3-1. (A) Physical map of the *phh* operon in *Pseudomonas aeruginosa*. The endonuclease restriction sites are shown at the top. The arrows indicate the position of the genes and the directions of transcription. Putative transcriptional terminators (*t* inside a circle) are indicated. The proteins encoded by the genes are as follows: *phhR*, σ^{54} transcriptional activator of the *phh* operon; *phhA*, phenylalanine hydroxylase; *phhB*, 4**a**-carbinolamine dehydratase; and *phhC*, aromatic aminotransferase. (B) Regeneration of the pterin cofactor for phenylalanine hydroxylase. The enzymes involved are indicated as follows: PhhA, phenylalanine hydroxylase; PhhB, 4**a**-carbinolamine dehydratase; and DHPR, dihydropteridine reductase.

carbinolamine dehydratase and regulatory activity as the dimerization cofactor of HNF1α. In this chapter, I report the results of studies aimed at elucidation of the extent and nature of the regulatory function of PhhB protein.

Materials and Methods

Bacterial Strains, Plasmids, Phage, and Media

The bacterial strains, plasmids, and phage used in this study are listed in Table 3-1. The LB and M9 formulations (Sambrook et al., 1989) were used as growth media for *E. coli* and *P. aeruginosa*. Pseudomonas isolation agar (Difco) was used for isolating *P. aeruginosa* knockout mutants. Additions of ampicillin (100 μg/ml), chloramphenicol (40 μg/ml), kanamycin (50 μg/ml), mercuric chloride (15 μg/ml), L-phenylalanine (50 μg/ml), and thiamine (17 μg/ml) were made as indicated. Agar was added at a final concentration of 2% (w/v) for preparation of solid medium.

Recombinant DNA Techniques

Molecular cloning and DNA manipulation including plasmid purification, restriction enzyme digestion, ligation, and transformation were conducted by standard methods (Sambrook *et al.*, 1989). DNA fragments were purified from agarose gel with a Geneclean kit (Bio 101). Electroporation (Invitrogen) was used for simutaneous transformation of *E. coli* with two compatible plasmids. Restriction enzymes, T4 DNA ligase, DNA-

Table 3-1. Bacterial strains, plasmids, and phages used in this study

Strain or plasmid	Relevant genotype or description	Source or reference
E. coli		
BL21(DE3)	F⁻ *ompT hsdS*$_B$ ($r_B^-m_B^-$) *gal dcm*; with DE3, a λ prophage carrying the T7 RNA polymerase gene	Novagen
DH5α	F⁻ Δ*lacU169 ϕ80dlacZΔM15 hsdR17 recA1 endA1 gyrA96 thi-1 relA1 supE44*	GIBCO/BRL
LE392	F⁻*e14*⁻*(McrA⁻)hsdR514*($r_k^-m_k^+$) *supE44 supF58 lacY1 or Δ(lacIZY)6 galK galT22 metB1 trpR55*	Sambrook *et al.*
JP2255	*aroF363 pheA361 pheO352 tyrA382 thi-1 strR712 lacY1 xyl-15*	Baldwin & Davidson
JS1	SP1313Φ(*phhA'-lacZ*)	This study
S17-1	[RP4-2(Tc:Mu)(Km:Tn7)Tra(incP)] *pro hsdR recA* Tp^r Sm^r	Simon *et al.*
SP1313	*zah-735*:Tn*10*Δ(*argF-lac*)*U169*Δ(*tyrR*)	Heatwole & Somerville
P. aeruginosa		
PAO-1	Prototroph	Holloway
JS101	PAO-1 *phhA*, Hg^r	Song & Jensen
JS102	PAO-1 *phhR*, Hg^r	Song & Jensen
JS103	PAO-1 *phhB*, Hg^r	This study
JS104	PAO-1 *phhC*, Hg^r	
Plasmids		
pUC18	Amp^r *lac'*IPOZ'	Yanisch-Perron *et al.*
pUC19	Amp^r *lac'*IPOZ'	Yanisch-Perron *et al.*
pACYC177	P15A replicon, Ap^r Km^r	Chang & Cohen

Table 3-1. (continued)

pET11b	T7*lac* promoter, *lacI*$^+$ Ap^r	Novagen
pET23	T7*lac* promoter, *lacI*$^+$ Ap^r	Novagen
pGEM-3Z	T7 promoter, Ap^r	Promega
pGST-DCoH	In-frame protein fusion of glutathione S-transferase and DCoH	Citron *et al.*
pJS10	*phhAB*, 2.5-kb *Hinc*II fragment cloned into pGEM-3Z behind the T7 promoter	This study
pJS11	*phhAB'*, 1.44-kb *Hinc*II-*Eco*RV fragment cloned into pACYC177	This study
pJS12	*phhAB*, 2.5-kb *Hinc*II fragment cloned into pACYC177	This study
pJS51	*Hinc*II-*Bam*HI fragment containing truncated *phhA'* cloned into pACYC177	This study
pJS51Z	*phhA'-lacZ* transcriptional fusion in pACYC177	This study
pJS63	*phh'ABC*, *Bam*HI-*Hin*dIII fragment cloned into pGEM-3Z behind the T7 promoter	This study
pJS72	*phhA*, PCR-generated fragment containing the native ribosome -binding site and PhhA-coding region cloned into pET23 behind T7*lac* promoter	This study
pJS95	PhhA overexpression vector; PhhA-coding region fused with T7 translational initiation signal at *Nde*I site of pET11b	This study
pJS96	PhhA overexpression vector; *phhA* fused with T7 translational initiation signal cloned into pUC19 behind *lac* promoter to constitutively overexpress PhhA	This study

Table 3-1. (continued)

pJS97	PhhA overexpression vector; *phhA* fused with T7 translational signal cloned into pTrc99A behind *trc* promoter	This study
pJS101	Hg^r-cassette, Ap^r	Song & Jensen
pJS105	*Hinc*II-*Bam*HI PCR fragment containing *phhA'* with a frameshift	This study
pJS105Z	*phhA'-'lacZ* protein fusion cloned into pACYC177	This study
pJZ9	*phhRABC*, Ap^r	Zhao *et al.*
pJZ9-3a	*phhAB*, Ap^r	Zhao *et al.*
pJZ9-4	*phh'ABC'*, Ap^r	Zhao *et al.*
pJZ9-5	*phhAB'*, Ap^r	Zhao *et al.*
pMC1871	*lacZ* protein fusion vector	Pharmacia
pTrc99A	*Trc* promoter, *lacI*$^+$ Ap^r	Pharmacia
pUFR004	ColE1 replicon, Cm^r Mob^+ *mob*P, *lacZ*α^+	DeFeyter *et al.*
Z1918	Promoterless *lacZ*, Ap^r	Schweizer
Phages		
λRZ5	λ*'bla 'lacZ lacY*$^+$	Resental *et al.*
λJS1	λΦ(*phhA'-lacZ*) *lacY*$^+$ *'bla*	This study

modifying enzymes (New England Biolab or Promega), Taq DNA polymerase (Perkin-Elmer), and Vent DNA polymerase (New England Biolab) were used as recommended by the suppliers.

Phenylalanine Hydroxylase Assay

E. coli JP2255(pJZ9-3a) was grown at 37°C in 500 ml of LB broth supplemented with ampicillin (100 μg/ml), and harvested at the late-exponential phase of growth. Cell pellets were resuspended into 8 ml of 10 m*M* potassium phosphate buffer (pH 7.4) containing 1 m*M* dithiothreitol (DTT), and the cells were disrupted by sonication. The resulting extract was centrifuged at 150,000 x *g* for 1 hr at 4°C. The supernatant was desalted using Sephadex G-25 and used as crude extract for enzyme assay. Phenylalanine hydroxylase (PhhA) was assayed by following tyrosine formation (Nakata *et al.*, 1979).

Phenylalanine Hydroxylase Stimulation Assay

4**a**-Carbinolamine dehydratase activity in *E. coli*(pJZ9-4) was assayed indirectly using the phenylalanine hydroxylase stimulation assay (Citron *et al.*, 1992). Reaction mixtures containing 30 mM potassium phosphate (pH 8.3), catalase (1 mg/ml), 100 μ*M* NADH, 1 m*M* phenylalanine, 20 μg dihydropteridine reductase, 14.4 μg rat liver phenylalanine hydroxylase, and 2.9 μ*M* 6,7-dimethyltetrahydropterin were incubated at 25°C. Approximately 1 min after the reaction was started, either buffer (control) or 15 μg of the *E. coli*

crude extract containing PhhB or GST-DCoH was added. The reaction was monitored at 340 nm for the oxidation of NADH by dihydropteridine reductase as quinonoid dihydropterin was recycled to tetrahydropterin.

Construction of *phhA:lacZ* Transcriptional and Translational Fusions

For the transcriptional fusion (*phhA'-lacZ*), the *Hinc*II-*Bam*HI fragment containing the upstream region of *phhA* was first cloned into pACYC177 that had been digested with *Hinc*II and *Bam*HI, creating pJS51. A *Bam*HI-cassette of a promoterless *lacZ* gene from the plasmid Z1918 was then inserted at the *Bam*HI site of pJS51 in the same orientation as *phhA* to create pJS51Z, which was used as a low-copy *phhA'-lacZ* fusion. A single-copy fusion λ(*phhA'-lacZ*) was obtained by transferring the *phhA'-lacZ* fusion from pJS51Z into λRZ5 following the procedure described by Yu and Reznikoff (1984).

For the translational fusion (*phhA'-'lacZ*), the *Hinc*II-*Bam*HI fragment containing the upstream region of *phhA* was generated by PCR with the upper primer 5'-GACAGAGCAGGTAGATGGCGTT-3', and the lower primer 5'GGGATCC**G**GCTCGTGGGGCAGGCCGA-3' (*Bam*HI site underlined). An extra guanine nucleotide (G in bold) was added in the lower primer to create the frameshift needed for an in-frame fusion at the *Bam*HI site to generate *phhA'-'lacZ*. The *Hinc*II-*Bam*HI fragment with the frameshift was inserted into the *Hinc*II-*Bam*HI site of pACYC177 to create pJS105, and a *Bam*HI-cassette

of truncated *'lacZ* from pMC1871 was inserted into pJS105 to create the translational fusion plasmid, pJS105Z.

β-Galactosidase Assay

β-Galactosidase activity was assayed under conditions of proportionality as described by Miller (1972), and specific activities are expressed in Miller units. The data are the results of at least two independent assays.

Construction of PhhA and PhhB Expression Vectors

To express PhhA protein, expression plasmids pJS72 and pJS95 were constructed. A PCR fragment containing the complete coding region of *phhA* and the native ribosome-binding site (RBS) was amplified with the upper primer 5'-CATGGAGTCCGT**ATG**AAAACGACGCA-3' (RBS underlined; ATG start codon in bold) and the downstream primer 5'-CTTGGTTGTCGCATGTGGGAGCGGCG-3', and cloned into pET23 behind the T7*lac* promoter to create pJS72. pJS95 was constructed by inserting the coding region of *phhA* into the translational fusion vector pET11b. The coding region was amplified by PCR with the upstream primer 5'-CCA**TATG**AAAACGACGCAGTACGTG-3' and the downstream primer 5'-CAAGTCTGGTTGTCGCATGTGGGAGCGGCG-3'. The upper primer was made with a built-in *Nde*I site (underlined) which allows fusion of *phhA* at the translational start site (ATG in bold) with the T7 translational initiation signals. To constitutively express the PhhA protein, the

phhA-coding region together with the upstream T7 translational start signals were excised from pJS95 as a *Xba*I fragment and cloned into pUC18 downstream of a *lac* promoter to create pJS96. The *Xba*I fragment was also cloned into pTrc99A downstream of the inducible *trc* promoter to create pJS97.

Two similar plasmids, pJS10 and pJS63, were constructed to express PhhB. The *Hinc*II fragment containing both *phhA* and *phhB* gene was inserted into pGEM-3Z to create pJS10, and the *Bam*HI-*Hin*dIII fragment containing both *phhB* and *phhC* was inserted into pGEM-3Z to create pJS63. The *phhB* gene was under the control of a T7 promoter in both plasmids.

Preparation of PhhB-specific Polyclonal Antiserum

PhhB was partially purified by anion-exchange and gel-filtration chromatography following the methods described by Zhao *et al.*, (1994). The partially purified PhhB was subject to SDS-PAGE (12%) and the gel was stained with Commassie blue R-250. The PhhB band was cut from the gel and used for the production of polyclonal antiserum in rabbits (Cocalico Biologicals, Inc., Reamstown, PA). Antiserum was purified by using an Econo-Pac protein A column (Bio Rad) and further absorbed with a total cell extract from the PhhB-deficient mutant JS103.

SDS-PAGE and Western Blot Analysis

SDS-PAGE (12% gel) was performed with the Mini-PROTEAN II cell (Bio-Rad) by the method of Laemmli (1970). Samples of

exponential-phase cells were collected by centrifugation, and the cell pellets were suspended in gel-loading buffer and heated at 100°C for 10 min. Samples of 5-10 μl were loaded onto two SDS-polyacrylamide gels. After separation of the proteins by electrophoresis, one gel was stained with Coomassie blue and the other gel was used for blotting. When crude extracts were used, equivalent amounts of protein were loaded into each lane. Western blots were performed according to Towbin *et al.* (1979). The proteins were electrophoretically transferred onto nitrocellulose membranes and reacted with the polyclonal antiserum at a dilution of 1:1000. Membranes were then incubated with secondary alkaline phosphatase-labelled anti-rabbit antibodies at a dilution (1:30,000) and developed by adding NBT and BICP as chromogenic substrates (Bibco BRL) for alkaline phosphatase.

Gene Inactivation

Both *phhB* and *phhC* were inactivated following the method described by Song and Jensen (1996). To generate the truncated '*phhB*' fragment (308bp), the upper primer 5'-ACCCAAGCCCATTGCGAAGCCTGCCG-3', and the lower primer 5'-GTGCGCGCCGCCATGATGAAATCGTT-3' were used. To generate the truncated '*phhC*' fragment (652bp), the upper primer 5'-GTCGAGCAGGAAACCACCAAGA-3', and the lower primer 5'-GTTGGCTACGCAGGTCGGTGAG-3' were used. Interruption of the *phhB*

or *phhC* gene in a Hg^r isolate was confirmed by Southern hybridization.

Southern Hybridization

Genomic DNA was extracted from the *P. aeruginosa phhB*⁻ strain by the method described by Silhavy *et al.* (1984). Southern hybridization was performed as described by Sambrook *et al.* (1989). The DNA was completely digested with *Eco*RI, separated by electrophoresis in 1% agarose gel, and transferred to a nylon membrane (Bio-Rad). The DNA was fixed by baking the membrane under vacuum at 80°C for 2 hr and hybridized at 42°C overnight with the truncated *'phhB'* (the same as used for gene inactivation) probes that had been labeled with biotin-14-dATP using a BioNick labelling system (GIBCO/BRL). The membrane was washed in 2X SSC (300 m*M* NaCl, 30 m*M* sodium citrate, pH 7.0) plus 0.1% SDS (twice for 3 min each time at room temperature), in 0.2X SSC plus 0.1% SDS (twice for 3 min each time at room temperature), and in 0.16X SSC plus 0.1% SDS (twice for 15 min each time at 50°C). The probes were detected with the BluGene nonradioactive nucleic acid detection system (GIBCO/BRL).

RESULTS

PhhB Has 4a-Carbinolamine Dehydratase Activity

PhhB is a homologue of an established 4**a**-carbinolamine dehydratase. To confirm that PhhB catalyzes the 4**a**-

carbinolamine dehydratase reaction, I used the phenylalanine hydroxylase stimulation assay where the utilization of 4**a**-carbinolamine limits the rate of the hydroxylation (Huang *et al.*, 1973; Citron *et al.*, 1992). Either PhhB or DCoH was able to stimulate the phenylalanine hydroxylase reaction in *E. coli* crude extracts where DCoH was used as a postive control (Fig. 3-2), indicating that PhhB protein has 4**a**-carbinolamine dehydratase activity. Furthermore, using the expression construct pJS63, PhhB protein was purified in the laboratory of Dr. June E. Ayling at the University of South Alabama and the 4**a**-carbinolamine dehydratase activity of PhhB was confirmed by direct assay (personal communication).

Complementation of Tyrosine Auxotrophy by *phhA* and *phhB in trans*

Both *phhA* and *phhB* are needed for functional complementation of *E. coli* tyrosine auxotrophy (Zhao *et al.*, 1994). If *phhB* functions as both a structural gene and a regulatory gene in a fashion that parallels the mammalian homologue, it would be expected to complement in the *trans* comfiguration with respect to *phhA*. A *trans*-complementation study was done in which *phhA* and *phhB* (or DCoH) were inserted into two compatible plasmids, pJS11 and pJZ9-4A (or pGST-DCoH), respectively. The results (Table 3-2) did indeed show that *phhB* was able to complement *E. coli* tyrosine auxotrophy *in trans* with respect to *phhA* and that mammalian DCoH was able

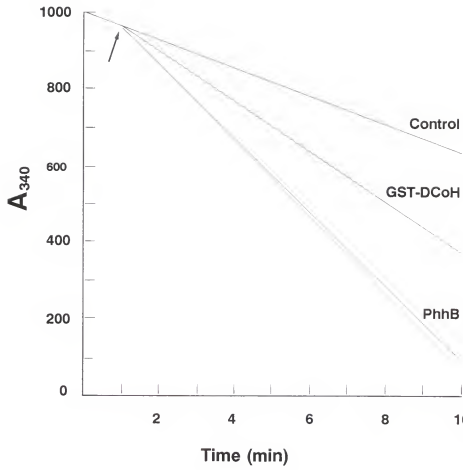


FIG. 3-2. Stimulation of phenylalanine hydroxylase activity by the addition (at the arrow) of a crude extract of *E. coli* JP2255 containing PhhB or GST-DCoH fusion protein. Approximately 1 min after the reaction was started, either buffer, or 15 μg of the crude extract containing PhhB, or GST-DCoH was added. The reaction was monitored at 340 nm for the oxidation of NADH by dihydropteridine reductase as quinonoid dihydropterin was recycled to a tetrabiohydropterin (see Fig. 3-1).

Table 3-2. Complementation of an *E. coli* Tyr^-[a] mutant by *phhA* and either *phhB* or *DCoH* gene *in trans*

Plasmid(s)	Relevent genotype	Ability to complement[b] *E. coli* Tyr^- mutant
pJS11	*phhA*$^+$	No
pJS12	(*phhAB*)$^+$	Yes
pJZ9-4	*phhB*$^+$	No
pJS11 + pJZ9-4	*phhA*$^+$ *phhB*$^+$	Yes
pGST-DCoH	*DCoH*$^+$	No
pJS11+pGST-DCoH	*phhA*$^+$ *DCoH*$^+$	Yes

[a] *E. coli* JP2255 (Phe^-Tyr^-) mutant was used as the host strain for this complementation study.

[b] *E. coli* JP2255 harboring various plasmids was plated on M9 + phenylalanine plates supplemented with appropriate antibiotics as selective agents.

to replace PhhB in the bacterial system. The results also ruled out any possible *cis* effect of *phhB* on the expression of *phhA*.

Expression of *phhA* in the Presence or Absence of *phhB*

The *trans*-complementation study confirmed that complementation of *E. coli* tyrosine auxotrophy by *phhA* requires the presence of *phhB*. To understand whether the requirement of *phhB* for the complementation was due to increased expression of *phhA* in the presence of PhhB, the expression of *phhA* in the presence or absence of *phhB* in *E. coli* JP2255 was studied through Western analysis (Fig. 3-3). A substantial level of PhhA was still detected when only *phhA* was present. The presence of either PhhB or DCoH *in trans* indeed increased the expression of *phhA*, but only by about 2-fold. Although this result indicated that PhhB may regulate the expression of *phhA*, much more than the relatively modest 2-3 fold reduction in PhhA level observed in the *phhB*$^-$ mutant was expected. This was because little or no PhhA had been detected in the absence of *phhB* (pJZ9-5) in *E. coli*, whereas a very high level of PhhA was produced in the presence of *phhB* (pJZ9-3a) (Zhao *et al.*, 1994). The inconsistency between the results of this study and that obtained by Zhao *et al.* (1994) was found to be due to the incorrectly reported orientation of the *phhA* insert in pJZ9-5. Sequencing the DNA insert in pJZ9-5 revealed that the orientation of the *phhA* gene was opposite to

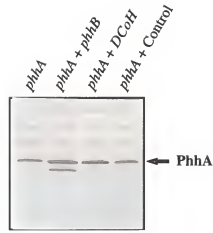


FIG. 3-3. Western blot analysis of PhhA expression in *E. coli* JP2255. Proteins in the whole cell lysates of JP2255 carrying various plasmids were separated by SDS-PAGE and reacted with rabbit anti-PhhA polyclonal antibodies. Plasmids containing the gene(s) shown above are as follows: *phhA*, pJS11; *phhB*, pJZ9-4; *DCoH*, pGST-DCoH. pUC18 was used as the control plasmid.

the *lac* promoter, rather than in the same orientation as reported by Zhao, *et al.* (1994).

PhhB Regulates Expression at the Post-transcriptional Level

To understand the regulatory role of PhhB in the expression of *phhA*, I constructed *phhA'-lacZ* transcriptional fusions in *E. coli* in both multi-copy form (pJS51Z) and a single-copy form [λ(*phhA'-lacZ*)]. In both cases, the presence of PhhB (pJZ9-4) or DCoH (pGST-DCoH) on a second plasmid did not result in a higher level of β-galactosidase as compared to the control (pUC18) (Table 3-3), indicating that neither PhhB nor DCoH functions at the transcriptional level. I then constructed the translational fusion *phhA'-'lacZ* (pJS105Z) (Table 3-3), and PhhB or DCoH was again provided *in trans* on a second plasmid. β-Galactosidase activity increased about two-fold, indicating that PhhB regulates the expression of *phhA* at the translational or post-transcriptional level. This level of activation by PhhB is consistent with the results obtained from Western-blot analysis in *E. coli*, where similarly modest levels of activation in *phhA* expression were observed.

Induction of The *phh* Operon by Phenylalanine in *P. aeruginosa*

Expression of both *phhA* and *phhB* were coordinately induced by phenylalanine in *P. aeruginosa* when grown on minimal fructose medium (Fig. 3-4). The induction process is

Table 3-3. Levels of *phhA* expression by *phhB* and *DCoH in trans*[a].

phhB or *DCoH in trans*	β-Galactosidase Activity[b]		
	pJS51Z	λ(*phhA'-lacZ*)	PJS105Z
pJZ9-4 (PhhB⁺)	180	13.2	13.1
pGST-DCoH (DCoH⁺)	191	14.1	10.4
pUC18 (control)	182	15.7	7.3

[a] Regulation of *phhA* expression was studied using *lacZ* as the reporter gene and β-galactosidase activities in transcriptional fusions pJS51Z (*phhA'-lacZ*) (multicopy) and λ(*phhA'-lacZ*) (single copy), and translational fusion pJS105Z (*phhA'-'lacZ*) (multicopy) was assayed in absence (pUC18) or presence of PhhB (pJZ9-4) and DCoH (pGST-DCoH).

[b] β-Galactosidase activities are reported in Miller units.

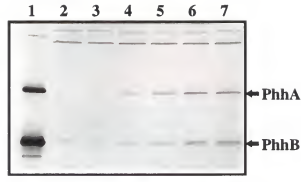


FIG. 3-4. Induction of the *phh* operon by phenylalanine hydroxylase in *P. aeruginosa*. Proteins in the whole cell lysates were separated by SDS-PAGE. Lane 1, *E. coli* JP2255 harboring pJS11 and pJZ9-4; lanes 2-7, samples taken after elapsed times of 0, 10, 30, 60, 90, 120 min, respectively, following addition of 100 μg/ml phenylalanine at zero time.

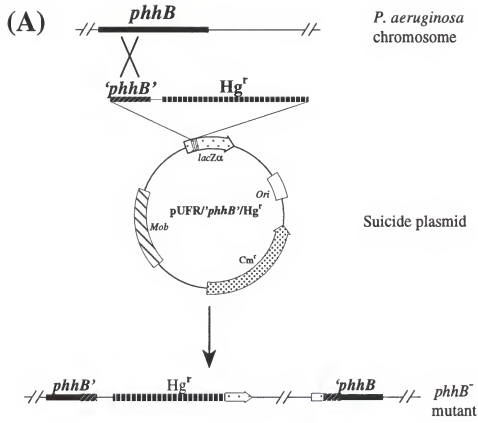
quite protracted and requires 90 minutes or more reach a maximum. A basal level of PhhB was expressed under non-inducing conditions as seen in lane 2 on the Western blot. It is unknown whether this is due to expression from a promoter upstream of *phhA* or from an internal promoter within the coding sequence of *phhA*. Although the latter interpretation comes to mind because no PhhA band is visualized at zero time, this could be due to differing sensitivities of the antibody probes.

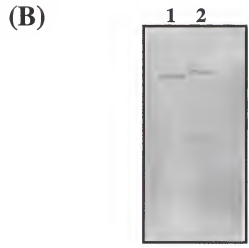
Effect of *phhB* Knockout in *P. aeruginosa*

The *phhB* gene in *P. aeruginosa* was inactivated by chromosomal insertion of the suicide plasmid pUFR/'*phhB*'/Hg^r through a single homologous crossover event (Fig. 3-5A). The insertional inactivation of the *phhB* gene in the resulting mutant was confirmed by Southern blot analysis (Fig. 3-5B).

The expression of PhhA in a *phhB*$^-$ background was examined by Western-blot methodology under fully induced conditions fostered by growth on LB media (Fig. 3-6). The PhhA level observed in the *phhB*$^-$ mutant was reduced about 2-3 fold compared to the wildtype parent. No PhhA was detected in *phhA*$^-$ mutant (negative control). The PhhB level was also checked at the same time. A low basal level of PhhB was detected in *phhA*$^-$ mutant when compared with that in the wildtype. No PhhB was detected in *phhB*$^-$ mutant (negative control).

FIG. 3-5. Inactivation of *phhB* in *P. aeruginosa*. (A) Schematic representation for insertional inactivation of the chromosomal phhB gene by the integration of the suicide plasmid pUFR/'*phhB*'/Hg^r through a single homologous crossover. The resulting Hg^r mutant does not contain a complete copy of *phhB* gene, but instead has two truncated copies of *phhB* gene. (B) Southern-blot analysis of chromosomal DNA from *P. aeruginosa* PAO-1 wildtype (lane 1) and mutant JS103 (lane 2). Chromosomal DNA was completely digested with *EcoRI* and probed at high stringency with the truncated '*phhB*' fragment of pUFR/'*phhB*'/Hg^r.


(A)
phhB
P. aeruginosa
chromosome
'phhB'
Hg^r
lacZα
Ori
pUFR/'phhB'/Hg^r
Mob
Cm^r
Suicide plasmid
phhB'
Hg^r
'phhB
phhB⁻
mutant


(B)
1 2

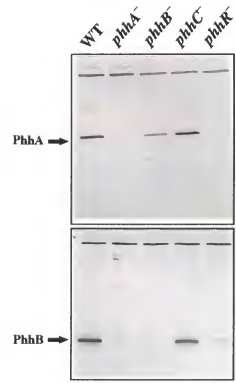


FIG. 3-6. Western blot analysis of PhhA and PhhB expression in *P. aeruginosa* PAO strains. Proteins in whole cell lysates were separated by SDS-PAGE and probed with rabbit anti-PhhA or anti-PhhB polyclonal antibodies.

The physiological effect of the *phhB* knockout mutant in *P. aeruginosa* was examined. Inactivation of *phhB* abolished the ability to grow on either phenylalanine or tyrosine as the sole carbon source. However, interpretation of this result is complicated by results obtained with a *phhC* knockout mutant which was also not able to grow on either phenylalanine or tyrosine as the sole carbon source. Since insertion of the suicide plasmid into the chromosome of *P. aeruginosa* is expected to create polar effects on the downstream genes in the operon (as indeed seen in *phhA* knockout where amount of PhhB expressed was dramatically decreased), it seems probable that the physiological effect observed in *phhB* knockout mutant is due to the polar effect on the expression of *phhC*.

Overexpression of PhhA and PhhB Proteins

Although PhhA was expressed to detectable levels in both *E. coli* (Fig. 3-3) and *P. aeruginosa* (Fig. 3-6) in the absence of *phhB*, initial attempts to express PhhA at high levels in the absence of PhhB in *E. coli* were unsuccessful. I then employed a T7 overexpression system (see *Methods*) to express PhhA in *E. coli* BL21(DE3) under induction conditions not requiring growth (Fig. 3-7A). When *phhA* was expressed from a native ribosomal binding site in pJS72, high PhhA levels were produced after IPTG induction for 3 h (Lane 3, Fig. 3-7B). When *phhA* was expressed from ϕ10 translational signals (pJS95), higher levels of PhhA was made after IPTG induction

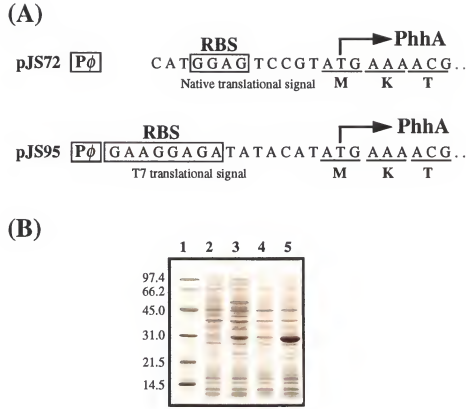


FIG. 3-7. Expression of PhhA using T7 expression system in *E. coli*. (A) Construction of PhhA expression plasmids, pJS72 (with the native translational signals) and pJS95 (with ϕ10 translational signals). (B) SDS-PAGE analysis of expressed PhhA protein. Proteins in whole cell lysate were separated by SDS-PAGE and stained with Coomassie blue. Lane 1, molecular weight markers; lanes 2&3, BL21(DE3) harboring pJS72, before and after 1 mM IPTG induction for 3 h at 30°C, respectively; lanes 4&5, BL21(DE3) harboring pJS95, before and after 1 mM IPTG induction for 3 h at 30°C, respectively.

for 3 h (Lane 5, Fig. 3-7B). PhhA produced using this T7 overexpression system in *E. coli* is active. Phenylalanine hydroxylase activity was assayed in crude extract of *E. coli* BL21(DE3) harboring either pJS72 or pJS95 after IPTG induction, and compared with activity present in *E. coli* JP2255 (pJS9-3a). Expression of *phhA* from ϕ10 translational signals resulted in over a five-fold increase in phenylalanine hydroxylase activity (Table 3-4).

PhhB protein was expressed very well from a *lac* promoter in pJZ9-4 by Zhao *et al.*(1994). We obtained an even higher level of PhhB by using the T7 overexpression system (Fig. 3-8). The pJS10 construct used contains both *phhA* and *phhB*. After IPTG induction, only PhhB was overexpressed and little PhhA was made (Lane 3, Fig.3-8). A similar phenomenon was observed with pJS63 construct containing both *phhB* and *phhC* where only PhhB protein was overproduced (Lane 5, Fig. 3-8). These results indicated that *phhB* has stronger translational signals and, therefore, is preferably translated over *phhA* and *phhC*. PhhB protein expressed is fully active, and has been purified and characterized from the latter construct by Ayling (unpublished results).

Expression of PhhA without PhhB in *E. coli* Has Growth Inhibitory Effects

Even though a low level of PhhA was produced from pJS11 in the absence of PhhB, it did not complement the *E. coli* tyrosine auxotrophy in JP2255. A possible explanation was that

Table 3-4. Phenylalanine hydroxylase activities in different expression clones[a]

Expression clones	Specific Activity (nanomoles/min/mg)
JP2255/pJZ9-3a	91.7
BL21(DE3)/pJS72	72.6
BL21(DE3)/pJS95	379.2

[a] Cells of *E. coli* JP2255 harboring pJZ9-3a were grown in LB broth at 37°C and harvested at late exponential phase; cells of BL21(DE3) harboring pJS72 or pJS95 were grown in LB broth at 37°C to O.D=1 and induced for 3 hr by addition of 1 mM IPTG. Crude extracts were used as the enzyme sources.

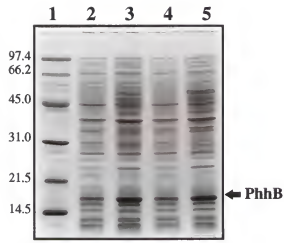


FIG. 3-8. Expression of PhhB using T7 expression system in *E. coli*. Proteins in whole cell lysates were separated by SDS-PAGE and stained with Coomassie blue. Lane 1, molecular weight markers; Lanes 2&3, BL21(DE3)/pJS10, before and after 1 mM IPTG induction for 3 h at 30°C, respectively. Lanes 4&5, BL21(DE3)/pJS63, before and after 1 mM IPTG induction for 3 h at 30°C, respectively.

the PhhA produced from pJS11 was not high enough to result in complementation. Thus, attempts were made to construct clones from which higher levels of PhhA could be produced to see whether complementation of *E. coli* tyrosine auxotrophy would then occur. Two constructs pJS96 and pJS97 were made with a *Xba*I fragment from pJS95 carrying *phhA* gene (fused with ϕ10 translational signals) cloned into pUC18 behind a *lac* promoter or into pTrc99A behind a *trc* promoter, respectively (Fig. 3-9). Both plasmids were found to be unstable and cells tended to lose the plasmid, especially with pJS96 where *phhA* is constitutively expressed at a very high level from the *lac* promoter. An elapsed time of several days was required to see pinpoint colonies with pJS96. *E. coli* carrying pJS97 was able to grow to a pinpoint colony overnight at 37°C without IPTG induction, while it took two or more days to see the pinpoint colonies on the plate with IPTG induction. These results indicated that high level of *phhA* expression in the absence of *phhB* triggers potent growth inhibition. Although the expression of *phhA* from the *trc* promoter on pJS97 has to be induced because of the presence of a *lacI*^{q} gene on the plasmid, a high level of PhhA is still produced without IPTG induction because the *trc* promoter is very strong and leaky.

Because PhhA was constitutively expressed from pJS96 at high level, the cells were not able to maintain the plasmid. Therefore, a high level of PhhA could not be overproduced in *E. coli* (pJS97) (Lane 1, Fig. 3-9B). Since a lower level of

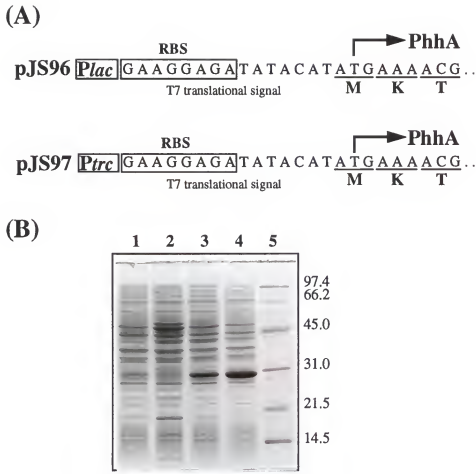


FIG. 3-9. Expression of PhhA in *E. coli* JP2255. (A) Construction of PhhA expression plasmids, pJS96 and pJS97. (B) SDS-PAGE analysis of PhhA expression. Proteins in whole cell lysates were separated by SDS-PAGE and stained with Coomassie blue. Lane 1, JP2255/pJS96; lane 2, JP2255/pTrc99A (control); lanes 3&4, JP2255/pJS97, before and after 1 mM IPTG induction for 3 h, respectively; lane 5, molecular weight markers.

PhhA was produced from pJS97 due to the presence of *lacI*^{q} gene one the plasmid, the cells carrying pJS97 were better able to maitain the plasmid, thus being able to overproduce PhhA after IPTG induction (Lane 4, Fig. 3-9B). Neither pJS96 nor pJS97 by itself was able to complement *E. coli* tyrosine auxotrophy. However, they were able to complement the auxotrophy when *phhB* was provided in trans on pJZ9-4 (data not shown). This result indicates that PhhB was able to remove the inhibitory effect imposed by overproduction of PhhA on the host cells. To assure that apparent growth inhibition was not due to excessive conversion of phenylalanine to tyrosine in the phenylalanine auxotrophy background of strain JP2255, the plasmids were moved to a prototrophic background. *E. coli* DH5α carrying either plasmid was found to develop only pinpoint colonies on LB + Amp plates without IPTG induction, confirming that expression of *phhA* at higher levels created a general inhibitory effect on the growth of *E. coli*.

Discussion

Regulatory Role of PhhB?

The mammalian PhhB homolog, DCoH, has both catalytic and regulatory functions. It was initially thought that *P. aeruginosa phhB* exerted an essential positive role in expression of *phhA* since constructs lacking *phhB* did not express *phhA*, as monitored by SDS-PAGE (Zhao et al., 1994).

Although *phhA* is in fact expressed in the absence of *phhB*, *phhB* does appear to exercise a positive regulatory role in the expression of *phhA* with a relatively modest effect of perhaps 2-3 fold. The transcriptional fusion approach seems to have eliminated the possibility of this regulation being at the transcriptional level, with the reservation that the experiments were performed in an *E. coli* background. I have shown that PhhR, the positive regulator of the *phh* operon in *P. aeruginosa*, does not interact properly with the *E. coli* σ^{54} machinery to activate *phhA* expression. But it is still possible that PhhB interacts with PhhR as a co-activator entity in the native *P. aeruginosa* organism. This would amount to a parallel with the mammalian system where DCoH is a co-activator for HNF1α (an upstream enhancer element that can be considered comparable to PhhR).

However, I have presented results to show that PhhB does enhance expression of PhhA at the post-transcriptonal level in *E. coli*. The 2-3 fold enhancement effects obtained correspond with the similar 2-3 fold magnitude of effect seen in *P. aeruginosa*, when comparing Western blots of PhhA in PhhB$^+$ and PhhB$^-$ backgrounds. If PhhB regulates at a post-transcriptional level, several possibilities envisioned include: (i) the sencondary structure of *phhA* mRNA might mask the ribosomal binding site. A stem-loop structure has been located in this area. Binding of PhhB in this region might disrupt the secondary mRNA structure and enhance translational initiation.

(ii) PhhB might bind *phhA* mRNA in the 3'region and protect against nuclease-catalyzed mRNA degradation. (iii) PhhB may complex with PhhA. This complex may protect PhhA from proteolysis. I have in fact obtained preliminary evidence for a PhhA-PhhB complex.

Rationale for Positive Regulatory Role of PhhB

When PhhA is highly expressed in the absence of PhhB, the *E. coli* host cells become subject to drastic growth inhibition. The reasonable explanation for this effect of PhhB is that removal of the inhibitory effect generated by PhhA reaction is direct result of 4**a**-carbinolamine dehydratase activity. It is known that in the absence of 4**a**-carbinolamine dehydratase activity, a 7-isomer of tetrahydrobiopterin is generated during the reaction catalyzed by phenylalanine hydroxylase (Davis *et al.*, 1992), and this 7-isomer is a potent inhibitor of phenylalanine hydrxoylase in the mammalian sytem. Since potent growth inhibition persists in wildtype *E. coli* backgrounds where overexpressed phenylalanine hydroxylase has no purpose, inhibition cannot be attributed to inhibition of phenylalanine hydroxylase by the 7-isomer. Two explanations accounting for general growth inhibition come to mind. (i) The reduced pterin cofactor of *E. coli* may be depleted because PhhB is not present for recycling. Consequently, some pterin-dependent enzymes that are essential for growth may become limiting. (ii)

Alternatively, the 7-isomer may be a potent inhibitor of a pterin-dependent enzyme needed for growth. Possible targets of inhibition could be dihydropteridine reductase or dihydrofolate reductase. The *E. coli* dihydropteridine reductase has been reported to possess broad specificity for pteridine compounds (Vasudevan *et al.*,1992).

When *phhA* was expressed at relatively low levels in *E. coli* no growth inhibition, or at least no severe growth inhibition occured. However, this PhhA was evidently not functional *in vivo* (in the absence of PhhB) because complementation of tyrosine auxotrophy in the presence of exogenous *L*-phenylalanine was unsuccessful. On the other hand, the joint presence of PhhA and PhhB readily allowed functional complementation. I concluded that PhhA is an essential target of the 7-isomer. At the low levels of PhhA expression in the absence of PhhB, the 7-isomer is generated and inhibits PhhA function--but not enough 7-isomer is produced to cause general growth inhibiton. At high levels of PhhA expression in the absence of PhhB, sufficient 7-isomer is produced to inhibit one or more enzymes essential for the growth.

With the above background, a rationale to explain a basis for selection of regulation of PhhA by PhhB is apparent. If it is correct that the 7-isomer generated from the carbinolamine pterin product of the PhhA reaction has general antimetabolite properties, then the significance of PhhB goes beyond its

catalytic capability. It also diverts the carbinolamine substrate from an undesirable nonenzymatic fate.

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BIOGRAPHICAL SKETCH

Jian Song was born on November 2, 1963, in Hebei Province, China. He completed his elementary and high school education in Xinhe County, Hebei Province. In 1980, he attended the Agricultural University of Hebei, where he majored in plant protection. He received his B.S. degree in 1984, then worked at the Institute of Plant Protection, Hebei Academy of Agricultural and Forestry Sciences until 1987. He was awarded a scholarship by Hebei Academy of Agricultural and Forestry Sciences to come to the Department of Entomology and Plant Pathology at the University of Tennessee, Knoxville for graduate study in 1988. He studied the interactions among the plant, aphid, and parasitoid under the supervision of Dr. Charles D. Pless. He received his M.S. degree in entomology in May, 1990. He then went to the Department of Entomology and Nematology at the University of Florida to continue graduate study toward a Ph.D. in entomology. He worked on insect toxicology under the supervision of Dr. Simon Yu until October, 1991. He then joined Dr. Roy A. Jensen's group at the Department of Microbiology and Cell Science in January, 1992 and studied regulation of phenylalanine hydroxylase system in *Pseudomonas aeruginosa*, and obtained his Ph.D. degree in May, 1997. He has accepted a postdoctoral position

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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